Origins of thyroid progenitors and tumor-initiating cells

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Front cover: ”Faith, hope and charity”
a triptyk of mouse embryo micrographs
by Ellen Johansson

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ISBN 978-91-629-0505-7 (PRINT)
ISBN 978-91-629-0506-4 (PDF)
This thesis is available at http://hdl.handle.net/2077/55395

Printed in Gothenburg, Sweden 2018
Printed by BrandFactory

This thesis is dedicated to my dear family

Γνώσεσθε τήν ἀλήθειαν,
καὶ ἡ ἀλήθεια ἐλευθερώσει ὑμας

You will know the truth, and the truth will set you free

– Gospel of John, chapter 8 verse 32

Κἂν ἔχω προφητείαν
καὶ εἰδῶ τὰ μυστήρια πάντα καὶ πᾶσαν τὴν γνῶσιν,
κἂν ἔχω πᾶσαν τὴν πίστιν ὥστε ὄρη μεθιστάνειν,
ἀγάπην δὲ μὴ ἔχω, ὀυθέν εἰμι

And if I have prophetic powers,
and understand all mysteries and all knowledge,
and if I have all faith, so as to remove mountains,
but have not love, I am nothing

– 1 Corinthians, chapter 13 verse 2

Abstract

The thyroid gland located in the anterior neck consists of two main cell types. First, the follicular cells that form the functional units, the follicles, in which thyroid hormones are produced and stored before release into the blood circulation; second, the parafollicular cells or C cells that produce calcitonin, a hormone that takes part in calcium regulation. These two cell types can give rise to different forms of cancer. Understanding basic mechanisms that govern the development and differentiation in the embryo may shed light on cell-specific mechanisms in tumor development.

In non-mammalian vertebrates neuroendocrine C cells retain in the ultimobranchial glands instead of being incorporated into the thyroid. Early quail-chick transplantation studies indicated that the C cells derive from the neural crest (i.e. are neuroectodermal), but this was not confirmed in mammals. In paper I, lineage tracing using a double fluorescent reporter (mTmG) showed that thyroid C cells in mouse embryos derive from pharyngeal endoderm instead of the neural crest. It was further shown that endoderm markers (Foxa1 and Foxa2) are dynamically regulated in invasive medullary thyroid carcinomas in humans. The actual entry of C cell precursors into the embryonic thyroid was investigated in paper II. Immunofluorescence and ultrastructural analysis with transmission electron microscopy indicated that the basement membrane of the ultimobranchial bodies is degraded before fusing with the thyroid primordium and that the process required Nkx2-1, a thyroid transcription factor. This suggested that migration and final parafollicular positioning of thyroid C cells is intrinsically regulated during development. In paper III, we modified an inducible mouse model of papillary thyroid cancer (the most common type of thyroid cancer). This mouse model is based on the Cre/loxP-system in which a $Braf^{V600E}$ mutation (constitutively activating the MAPK pathway) is conditionally activated only in thyroid follicular cells upon induction with tamoxifen. We discovered occurrence of sporadic Cre activity in the absence of tamoxifen and that microtumors developed clonally with functionally normal thyroid follicles side by side. Eventually, multifocal papillary thyroid carcinomas of different phenotypes (classical, tall-cell, hobnail, cystic and solid variants) developed within the same gland. Thus, this model enabled the detailed study of different stages in tumor development under conditions that closely resemble tumor development in humans. In paper IV, $TgCre;Braf^{V600E}$ mice were recombined with the mTmG reporter to trace mutant cells before overt tumorigenesis. A great diversity in proliferation rate among primary GFP-labeled cells that rarely developed into microtumors suggested the possibility of oncogene-induced senescence. Treatment with vemurafenib, a specific inhibitor to mutant Braf, inhibited focal tumorigenesis at an early stage, suggesting feasibility of the model in drug testing.

Keywords: Thyroid gland, thyroid cancer, mouse model, developmental biology, lineage tracing

ISBN 978-91-629-0505-7 (PRINT)
ISBN 978-91-629-0506-4 (PDF)

Sammanfattning på svenska

Sköldkörteln (tyreoidea) är ett hormonproducerande organ på halsen vid struphuvudet. Körteln byggs upp av små runda enheter som kallas folliklar och som består av follikelceller och C-celler. Follikelcellerna producerar sköldkörtelhormon för ämnesomsättningen och C-cellerna producerar hormonet calcitonin, som deltar i kroppens kalciumreglering. Sköldkörtelns celler har olika embryonala ursprung. Att känna till hur fosterutvecklingen går till i detalj är viktigt för att förstå grundläggande egenskaper hos cellerna i ett organ men också för att förstå mekanismer som är inblandade i tumörutveckling och som orsakar cancer.

Tidigare trodde man att C-cellerna under fostertiden bildas i den så kallade neurallisten varifrån de vandrar till den så kallade gälbågsapparaten i halsen på embryot, för att så småningom inkorporeras i sköldkörteln. I avhandlingens första delarbete visas att C-cellerna i själva verket kommer från endodermet, som är ett annat groddblad i embryot. Vidare visas att endodermala markörer finns i tumörer (medullär thyroideacancer) som bildas från muterade C-celler hos människa. Tumörcellerna återtar således vissa egenskaper som kännetecknar embryonala celler. I avhandlingens andra arbete ges en detaljerad beskrivning av hur C-cellernas förstadier bildas och hur cellerna förvärvar de egenskaper som kännetecknar dem normalt och möjligen efter tumöromvandling. Särskilt intresse ägnas basalmembranen som förankrar epitelcellerna och under sköldkörtelns utveckling utgör barriär mellan de olika celltyperna.

Förutom MTC finns sköldkörtelcancer även av papillär (PTC), follikulär (FTC) och anaplastisk typ (ATC). För att cancer ska uppstå krävs genetiska förändringar i en cell på ett sätt som leder till att cellen delar sig på ett okontrollerat sätt. Omkring hälften av alla patienter med PTC har en specifik mutation (BRAFV600E) som orsakar tumörsjukdomen. Med genteknik kan man aktivera just denna mutation i alla follikelceller vilket leder till kraftig tumörväxt. Detta skiljer sig dock från det vanliga förloppet hos människa där de allra flesta follikelceller saknar mutationen. I avhandlingens tredje delarbete presenteras en musmodell där vi visar hur Braf-mutationen kan begränsas till ett mindre antal follikelceller, vilket mer ger en mer realistisk modell av tumörutvecklingen hos människa. Det fjärde delarbetet utvecklar nämnda musmodell ytterligare och beskriver tidiga skeenden i tumörutvecklingen. I detta arbete kombineras musmodellen med en fluorescerande reporter där samtliga celler i musen bildar ett protein som lyser rött i fluorescensmikroskop. Med samma genteknik som aktiverar mutationen kan cellerna fås att istället bilda ett grönt färgämne, vilket gör att de celler som både har bytt färg och aktiverat mutationen enkelt kan identifieras och tumörer som växer från dessa tumörinitierande celler kan studeras redan innan de har gett upphov till egentlig cancer.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Ellen Johansson**, Louise Andersson, Jessica Örnros, Therese Carlsson, Camilla Ingeson-Carlsson, Shawn Liang, Jakob Dahlberg, Svante Jansson, Luca Parillo, Pietro Zoppoli, Guillermo O Barila, Daniel L Altschuler, Daniel Padula, Heiko Lickert, Henrik Fagman, Mikael Nilsson
Revising the embryonic origin of thyroid C cells in mice and humans
Development 2015(142):3519-28

II. **Ellen Johansson**, Shawn Liang, Henrik Fagman, Pina Marotta, Mario De Felice, Bengt R Johansson, Mikael Nilsson
Guidance of parafollicular cells (C cells) to the embryonic thyroid involves remodeling of basement membrane
Manuscript

III. Elin Schoultz, **Ellen Johansson**, Iva Jakubikova, Shawn Liang, Therese Carlsson, Bengt R Johansson, Henrik Fagman, Konrad Patyra, Jukka Kero, Martin Bergö, Mikael Nilsson
Follicular origin of tumor heterogeneity in a mouse model of sporadic papillary thyroid cancer
Manuscript

IV. **Ellen Johansson**, Carmen Moccia, Henrik Fagman, Mikael Nilsson
Tracing tumor-initiating cells in Braf^{V600E}-induced thyroid cancer
Manuscript

Papers not included in this thesis:
Shawn Liang, **Ellen Johansson**, Guillermo Barila, Daniel L Altschuler, Henrik Fagman, Mikael Nilsson
A branching morphogenesis program governs embryonic growth of the thyroid gland
Development 2017(145): p. dev146829
Ellen Johansson, Tobias E Holmin, Bengt R Johansson, Magnus Braide
Improving near-peer teaching quality by educating teaching assistants: an example from Sweden
Anatomical Sciences Education Feb 18, 2018 Epub ahead of print

Content

Abbreviations

APUD	Amine precursor uptake and decarboxylation
ATC	Anaplastic thyroid cancer
Cre	Causes recombination
Dapi	4'6 diamidino-2-phenylindole dihydrochloride
DTC	Differentiated thyroid cancer
EMT	Epidermal to mesenchymal transition
ERK	Extracellular singal-regulated kinase
Foxa1	Forkhead homeobox gene A1
Foxa2	Forkhead homeobox gene A2
FTC	Follicular thyroid cancer
loxP	Locus of x-over of bacteriophage P1
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MTC	Medullary thyroid cancer
mTmG	Membrane-bound Tomato/membrane-bound GFP
NC	Neural crest
Nkx2-1	NK homeobox gene 2-1
OIS	Oncogen-induced senescence
Pax8	Paired homeobox gene 8
PDTC	Poorly differentiated thyroid cancer
PTC	Papillary thyroid cancer
PTEN	Phosphatase tensin homologue
RAF	Rapidly growing fibrosarcoma
RET	Rearranged upon transfection
RTK	Receptor tyrosine kinase
SDG	Soli Deo gloria, glory to God alone
Sox17	SRY–related HMG-containing box gene 17
T3	Triiodothyronine
T4	Tetraiodothyronine
Tg	Thyroglobulin
Tam	Tamoxifen
TPO	Thyroperoxidase
TSH	Thyroid stimulating hormone
TTF-1	Thyroid transcription factor 1
UB	Ultimobranchial body

”There are only two ways to live your life.
One is as though nothing is a miracle.
The other is as though everything is a
miracle”

Albert Einstein
(1879–1955)

1 Preface

PREFACE

In this thesis I will describe and summarize the scientific work that I have done during my time as a PhD student. Working with science is a journey that often leads you into roads that you could not foresee. When I started my journey, I had not much of an idea where it would take me. Now it is time to sum up and put together different pieces that form a mosaic. The projects described cover different aspects of the development of the thyroid gland and events that lead to cancer. Findings from tracing studies of embryonic development and from a novel mouse model for studies of papillary thyroid cancer, will be presented. The summarizing chapters will introduce important concepts to the reader and give an overview of the research field. Methods that have been used will be explained and discussed. Finally, a short summary of the results and conclusions of the entire PhD project will be presented. The separate works that form the basis of this thesis are appended and indexed by Roman numerals.

”New York, the nation’s thyroid gland”

Christopher Morley
(1890-1957)

2 The thyroid gland

THE THYROID GLAND

Often resembled to a butterfly, the thyroid gland (illustrated in Figure 1) is a bilobed gland located in the anterior neck, close to the thyroid cartilage, from which it got its name (Wharton, 1656). The two lobes are connected by the isthmus. The thyroid gland produces several hormones from two distinct cell types: the follicular cells (thyrocytes) and the C cells. The follicular cells produce mainly the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) and the C cells produce calcitonin (Brent, 2012; Copp, 1992).

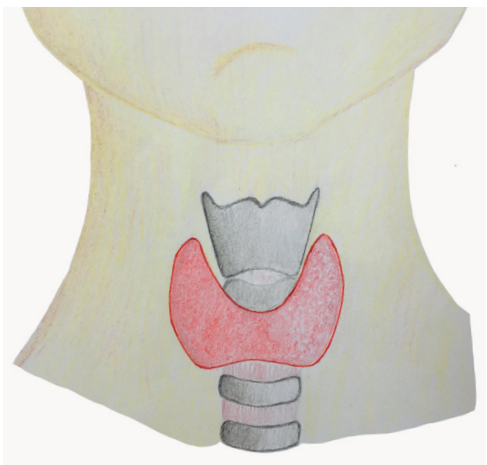

Fig 1. The thyroid gland (red) in front of the trachea.

2.1 THE THYROID FOLLICULAR EPITHELIUM

The follicle is the functional unit of the thyroid and it forms a spherical structure with follicular cells surrounding a lumen filled with colloid, see Figure 2. The follicular cells are organized in an epithelium with the apical side facing the lumen and the basal side resting on an enveloping basement membrane (Santisteban, 2005). This section will describe in more detail different features of the thyroid epithelium and its important role in producing and storing thyroid hormones.

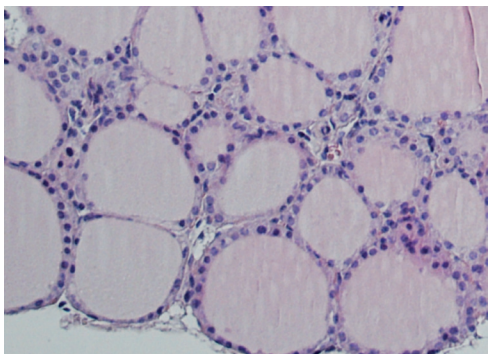

Fig 2. Micrograph of section from a mouse thyroid lobe. Follicles are formed by cells (purple nuclei) that surround colloid-filled lumina (pink).

2.1.1 Apical-basolateral polarity and cell-cell/matrix interactions

An important feature of any epithelium is polarity of the epithelial cells. The cell membrane of an epithelial cell can be divided into two distinct domains: the apical membrane, facing a lumen, and the basolateral membrane facing neighboring cells and the circulation (Ericson & Nilsson, 1992; Overeem, Bryant, & van Ijzendoorn, 2015). The two domains are separated by tight junctions to form a barrier that segregates apical and basolateral proteins and also enables strict control of the passage of different compounds between two separated compartments (Ojakian, Nelson, & Beck, 1997). In the case of the thyroid, these compartments are the follicle lumen and the extrafollicular space. On the apical side there are typically microvilli increasing the surface (Sauvanet, Wayt, Pelaseyed, & Bretscher, 2015). The thyroid epithelium displays all these features of polarity, exemplified in Figure 3.

Another difference between the two membrane domains is the distribution of membrane-bound receptors and other proteins. For example, follicular cells receive signals from circulating thyroid stimulating hormone (TSH), and the TSH receptor is thus located in the basolateral membrane (Beau et al., 1997).

Cells in an epithelium are polarized not only with respect to different plasma membrane constituents but also regarding the distribution of intracellular components. For example, the Golgi complex is normally located on the apical side of the nucleus (Bornens, 2008; Ojakian et al., 1997). Also the centrosome of a polarized cell shows an apical location, where it organizes microtubules, anchoring the apically located cilium (Carvajal-Gonzalez Jose, Mulero-Navarro, & Mlodzik, 2016). In paper II, antibodies against proteins associated with the Golgi complex and with the centrosome were used to monitor dynamic changes in epithelial polarity during embryonic development.

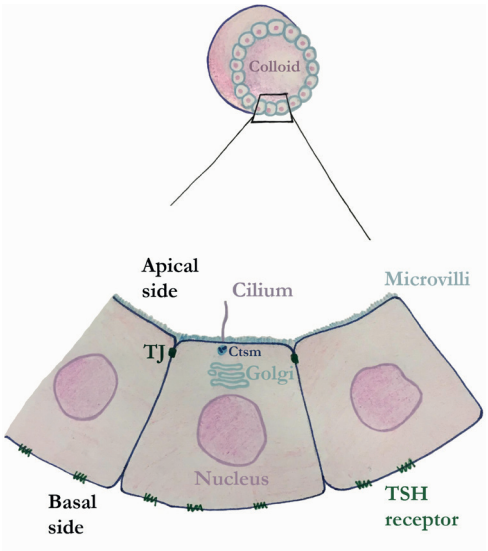

Fig 3. A thyroid follicle and a part of its epithelium with characteristic features.
Ctsm=centrosome, TJ=tight junction

Cell adhesion molecules (CAMs) are important in keeping epithelial cells closely together and maintaining the epithelial integrity (Näthke, Hinck, & Nelson, 1993). E-cadherin is a CAM that mediates calcium-dependent cell-cell adhesion and it is generally expressed in epithelia (C. Yoshida & Takeichi, 1982). See example of E-cadherin stai-

ning in Figure 4.

The expression of E-cadherin is lost in epithelial-mesenchymal-transition (EMT), an event that occurs normally during embryonic development, but pathologically in tumor progression and invasion. EMT is required to re-organize embryonic cells in the forming of complex tissues, but after reaching their final position, epithelial cells should be more or less stationary and regain their epithelial properties. When tumor cells lose their cell-cell adhesion and achieve migratory properties involving EMT (Chaffer, San Juan, Lim, & Weinberg, 2016), they can infiltrate locally, or enter the blood stream and metastasize to distant locations in the body. This is a hallmark of malignant tumor disease and a strongly negative prognostic factor (Guarino, Rubino, & Ballabio, 2007).

Thyroid cells express E-cadherin not only in the follicular state but also during development (H. Fagman, Grände, Edsbagge, Semb, & Nilsson, 2003). In papers I-III, immunostaining for E-cadherin was used to study its expression in embryonic development and in tumors.

Cells interact with their neighbors as well as with the surrounding matrix. The basement membrane (BM) underlying all epithelia is the closest layer of extracellular matrix (ECM) that provide mechanical support and a solid ground for the epithelia to rest on. BMs form a web of bioactive ECM proteins such as laminin, type IV collagen, nidogen and perlecan that influence cell properties (Yurchenco, 2011). The presence of a BM is thus important in maintaining the apical-basal polarity of epithelial cells. For example, adding BM constituents on the apical side of an epithelium can even reverse the polarity of the cells (O'Brien et al., 2001). BMs are delicate structures that are not visible in a light microscope unless some staining is used. In paper II immunostaining for the BM protein laminin, and electron microscopy were used to study the BM dynamics during embryonic development of the thyroid. See example of laminin staining in Figure 4.

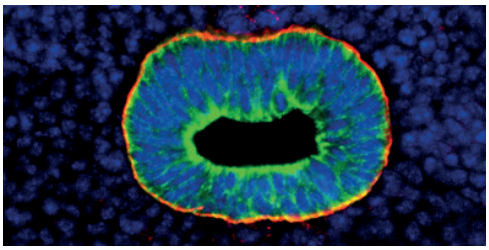

Fig 4. Example of an epithelium surrounding a lumen. All epithelial cells express E-cadherin (green) and the basal side rests on a basement membrane (red). Cell nuclei are blue

Laminin is a BM protein that forms a heterotrimer of α-, β-, and γ-subunits, illustrated in figure 5. The subunits form a coil with one end binding to the cellular surface and the other end forming three arms that bind to neighboring laminins and build up a network to which other BM constituents assemble (Hohenester & Yurchenco, 2013). The binding of laminin to cell surface receptors, e.g. dystroglycan, is important for the establishment of cell polarity and epithelial phenotype (Li, Edgar, Fässler, Wadsworth, & Yurchenco,

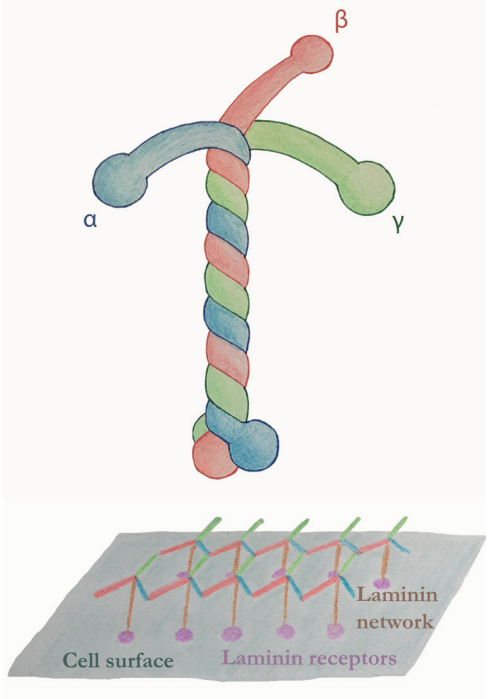

Fig 5. Upper: Laminin, a heterotrimer formed by three subunits. Lower: Laminin proteins forming a network on a cell surface.

2003). Conversly, impaired or altered binding functions of laminin may promote EMT and invasiveness (Giannelli, Bergamini, Fransvea, Sgarra, & Antonaci, 2005; Zeisberg & Neilson, 2009).

2.1.3 Uptake and transport of iodine

Iodine is a chemical element with atom number 53. In its gaseous state it forms a purple gas, hence the name from the Greek word ἰώδης, meaning 'purple'. Iodine is a relatively scarce element, and the ability to concentrate it is therefore crucial for all organisms that use it for hormone production. Iodide (I^-) is the reduced form of iodine that is transported and metabolized in cells (Wahab, 2009).

To achieve adequate concentrations of iodide, the thyroid needs a mechanism for iodide uptake, formerly referred to as iodide trapping (Wolff, 1964). This is provided by the sodium-iodide-symporter (NIS) that is located in the basolateral plasma membrane and transports iodide into the cell against its concentration gradient (Dai, Levy, & Carrasco, 1996; Dohán et al., 2003; Portulano, Paroder-Belenitsky, & Carrasco, 2014). After uptake, iodide is transported across the apical cell membrane and released into the colloid. The mechanism of apical I^- efflux, which is a regulated process (M. Nilsson, Björkman, Ekholm, & Ericson, 1990) is not fully understood but transporters as pendrin and anoctamin are thought to contribute (Silviera & Kopp, 2015; A. Yoshida et al., 2002).

2.1.2 Thyroid hormone production

The thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) are important for many metabolic processes in the body that include energy expenditure. The numbers 3 and 4 refer to the number of iodine atoms bound to the hormone molecule (Gross & Pitt-Rivers, 1953; Harington, 1926). See Figure 6 for chemical structure.

T_3 is biologically more active than T_4 and if there is an increased need of thyroid hormone supply, T_4 can be peripherally converted into T_3. In target cells, thyroid hormones bind to the thyroid hormone receptors that activate or repress transcription of a multitude of target genes (Brent, 1994).

The actual thyroid hormone synthesis (illustrated in Figure 7) takes place

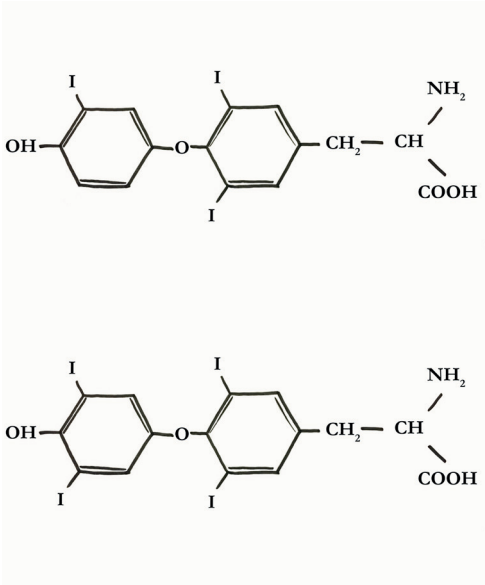

Fig 6. Thyroid hormones.
Upper: triiodotyronin, T_3 Lower: thyroxine, T_4

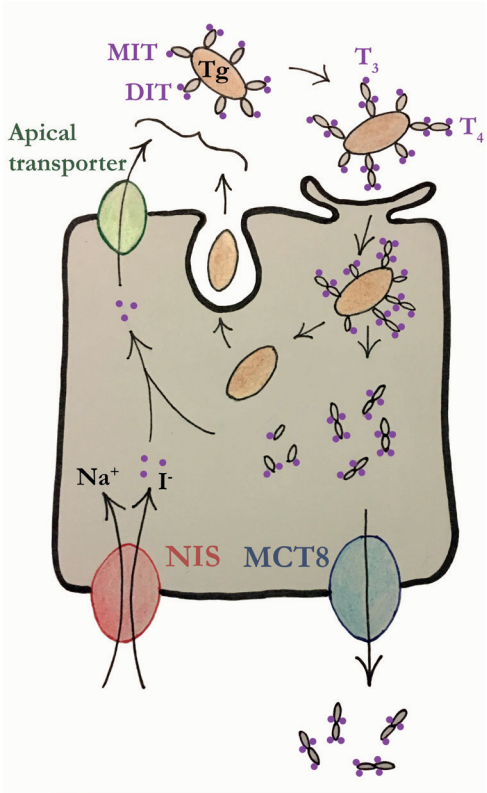

Fig 7. Synthesis of thyroid hormones.Tg (thyroglobulin, in brown) is produced by a follicular cell and transported by exocytosis into follicular lumen. Iodide (purple) is transported into the follicular cell by NIS (red) and then by an apical transporter (green) into the lumen, where it is coupled to tyrosine residues (oval-shaped) on Tg to create monoiodotyrosine (MIT) and diiodotyrosine (MIT). Next, MITs and DITs are combined to create thyroid hormones, T_3 and T_4. Tg is then transported back into the cell by endocytosis. MIT, DIT, T_3 and T_4 are released from Tg. Iodide from MIT and DIT are recycled in hormone synthesis. T_3 and T_4 are released into blood stream by the transporter MCT8 (blue).

extracellularly at the apical cell surface (Brix, Linke, Tepel, & Herzog, 2001; Ekholm, 1981). Thyroglobulin (Tg) and thyroperoxidase (TPO) are two important proteins in hormone synthesis that unlike NIS are expressed exclusively in the thyroid and only in follicular cells. Tg is a giant molecule (molecular weight around 330 kDa) that is synthesized by the follicular cells and released into the follicular lumen by exocytosis (Di Jeso & Arvan, 2016). TPO is a membrane-bound protein that iodinates and couples oxidized iodine to tyrosine residues on Tg in the presence of hydrogen peroxide (H_2O_2) (Corvilain, Van Sande, Laurent, & Dumont, 1991), which is produced by dual oxidase 1 and 2 (DuOX1/2), also at the apical membrane (Dupuy, Virion, Ohayon, Kaniewski, & Pommier, 1991; Massart et al., 2011). Monoiodotyrosine (MIT) and diiodotyrosine (DIT) are primarily produced. The coupling of MITs and DITs produces triiodothyronine (T_3) and thyroxine (T_4) (Malthiéry et al., 1989).

Iodinated Tg is stored in the lumen at a very high protein concentration, thus

forming the characteristic colloid. The follicular cells continuously internalize colloid by apical endocytosis to transport Tg into the follicular cell. Internalized Tg is degraded by lysosomal action, and T_3 and T_4 become released into the cytoplasm (Rousset et al., 1989). They are then available for transport into the blood stream that recently was found to be actively mediated by the monocarboxyl transporter 8, MCT8, located at the basal membrane of the thyroid epithelium (Bernal, Guadano-Ferraz, & Morte, 2015). Otherwise, MCT8 is primarily appreciated for its pivotal role in supplying the brain with thyroid hormones required for normal neurogenesis; patients with MCT8 inactivating mutations suffers from severe neurological impairments.

2.1.4 Regulation of thyroid hormone, growth and function

Thyroid hormone blood level is maintained by the pituitary gland, which releases thyroid stimulating hormone (TSH) in response to low levels (Magner, 1990). TSH has several effects on the thyroid. It stimulates the expression of thyroid genes (NIS, TPO, Tg and more) and increases NIS-mediated iodide uptake, TPO-mediated iodination, H_2O_2 production, and endocytosis of Tg. TSH thus stimulates almost all functions of the follicular cells aiming to increase thyroid hormone production and release.

Elevated levels of TSH also have a growth stimulating effect on the thyroid (Dumont, Lamy, Roger, & Maenhaut, 1992; T. Kimura et al., 2001). Clinically, enlargement of the gland regardless of the underlying reason is designated as goiter. Globally, the most common cause of goiter is iodine-deficiency. In these cases, the thyroid cannot produce sufficient amounts of thyroid hormones and this causes elevation of circulating TSH that stimulates thyroid cell proliferation, in some cases with growth of the gland to very large dimensions (Knobel, 2016). The mitogenic effect of TSH is mediated by activation of the cyclic AMP/protein kinase A pathway (Dremier et al., 2006). Under physiological conditions with normal thyroid hormone levels, thyroid cells replicate very slowly (Coclet, Foureau, Ketelbant, Galand, & Dumont J, 2008; Saad et al., 2006).

TSH stimulation causes morphological changes of the thyroid epithelium unrelated to hyperplasia. In follicles that are not active, the epithelium is generally flat and the lumen is large due to retention of colloid. When cells are stimulated by TSH, as a consequence of increased endocytosis, the amount of colloid is gradually reduced. Also, changes of shape make the cells more cuboidal. Active and inactive follicles can thus be discerned by different morphology and ratio between colloid and surrounding epithelium (Gérard et al., 2002). The follicular structure was an important parameter to evaluate in the mouse tumor models studied in papers III and IV.

2.2 C CELLS

The neuroendocrine cells of the thyroid were first named parafollicular cells by Nonidez (Nonidez, 1932) and later C cells by Pearse, not as an acronym of their hormonal product, but because the follicular cells were then called acinar or A cells and the endothelial cells of the thyroid were called B cells (Pearse, 1966). Thyroid C cells are scattered in the gland with predominance in the center of the lobes, reflecting their developmental origin, which will be discussed in detail in another chapter. Most often the C cells are found as a part of the follicles, invested by the same BM as the follicular cells, but they can also give rise to so-called solid cell nests (SCN) (Cameselle-Teijeiro et al., 1994; Harach, 1987). SCNs are considered to be remnants of the ultimobranchial epithelium from which the C cells derive. They can present a diagnostic problem since they could be mistaken for microcarcinomas and their potential to malignify has been a matter of debate (Manzoni et al., 2015).

The C cells are often polygonal in shape. They can be recognized by their expression of calcitonin, which can be easily detected by immunostaining. Ultrastructurally, they are characterized by the presence of electron dense granules in the cytoplasm typical of neuroendocrine cells (Ericson, 1968).

Calcitonin is a hormone that participates in regulating the levels of calcium in the body. However, in humans this function is redundant to the function of the parathyroid glands and calcitonin substitution is not necessary in patients who have undergone thyroidectomy (Davey & Findlay, 2013).

Nonetheless, calcitonin is an important serum marker to confirm a conspicuous thyroid carcinoma of C cell origin before the tumor identity is determined histologically and to monitor cancer relapse. Experimentally, calcitonin is used to differentiate C cells from others in both embryonic and adult thyroid. Papers I and II of the thesis focused on C cell development in mammals using mouse embryos as a model.

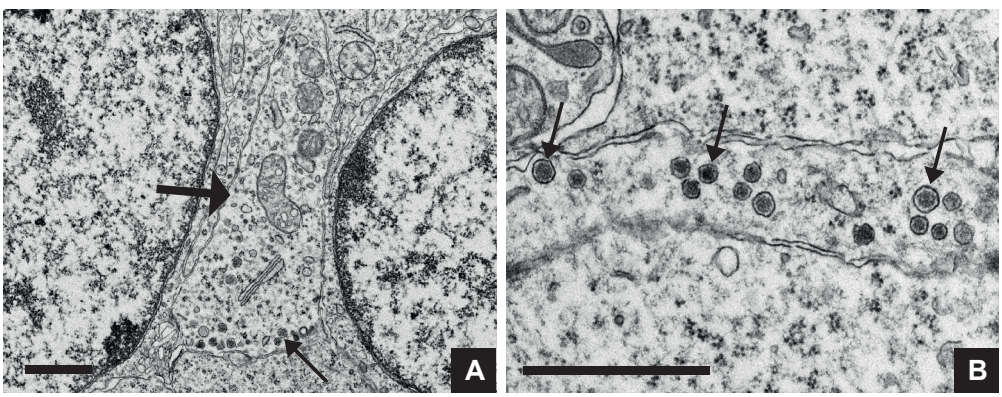

Fig 8. Electron micrographs of C cells. Large arrow in A shows a C cell between two follicular cells. Small arrows show electron dense granula in the cytoplasm. Scale bars: 1 μm

”I’ve always believed in the adage that the secret of eternal youth is arrested development”

Alice Roosevelt Longworth
(1884–1980)

3 Thyroid development

Thyroid Development

Much knowledge about how organs develop has come from the studies of animal models, such as mouse, chick and zebrafish (Castro-González, Ledesma-Carbayo María, Peyriéras, & Santos, 2012). These models recapitulate to different extent the embryonic development in humans. There are also studies where human embryos from legal abortions are used. Obviously, the representativeness of such studies cannot be controlled and they are also limited by problems of availability, not to mention the difficult ethical controversies that arise in human embryo studies. However, comparative studies on organogenesis in human and mouse embryos, strongly indicate that the developmental processes of many organs, including the thyroid, are very similar in the two species (Krishnan et al., 2014; Trueba et al., 2005; Xue et al., 2013).

Animal models have thus become important to perform detailed investigations of molecular mechanisms that govern embryonic development. They also provide opportunities to study the effects of genetic alterations with bearing on the understanding of dysgenesis leading to congenital malformations in children. This chapter will give an overview of the embryonic development of the head and neck region, especially regarding the thyroid primordia, and also describe the function of some genes that have proven important in regulating the development of the thyroid gland (Fernández, López-Márquez, & Santisteban, 2014).

3.1 THE PHARYNGEAL APPARATUS

The pharyngeal apparatus is formed along the anterior part of the gut tube (Frisdal & Trainor Paul, 2014; Graham, 2003). The pharyngeal arches are bulging segmental structures in the mesenchyme lateral to the gut epithelium. See Figure 9 for illustration. The arches have a dorso-ventral direction and are separated by pharyngeal pouches internally, and pharyngeal clefts externally. In fish, the branchial or gill arches are homologous to the pharyngeal arches, and occasionally

branchial refers to distinct derivatives of these transient structures. All three germ layers (ectoderm, mesoderm and endoderm) contribute to the pharyngeal apparatus and different parts of the endoderm are destined to form a number of glands in the developing fetus. The developmental course is mostly similar in mouse and human, but is naturally much more rapid in the mouse embryo (Gillam & Kopp, 2001). Unless otherwise specified, the following description will refer to the developmental events that are shared in mouse and human.

In each pharyngeal arch there is a pharyngeal arch artery (PAA) running from the aortic sac to the dorsal aorta. Similar to the arches and pouches, the PAAs are transient vessels that develop in cranial to caudal direction and all of them are not present at the same time. Parts of them will contribute to definitive vessels in the head and neck region (Rana, Sizarov, Christoffels, & Moorman, 2014).

The arches also get contribution of cells derived from the neural crest (NC). NC cells (NCC) that originally emigrate from the neural fold during formation of the neural tube. From this location in the dorsal part of the embryo some NCCs colonize the pharyngeal arches. Eventually, they give rise to skeleton, connective tissue and nerves (Graham, Begbie, & McGonnell, 2004). This is why cranial NCCs also are named ectomesenchymal referring to their (neuro)ectodermal origin.

The mesodermal parts of the arch

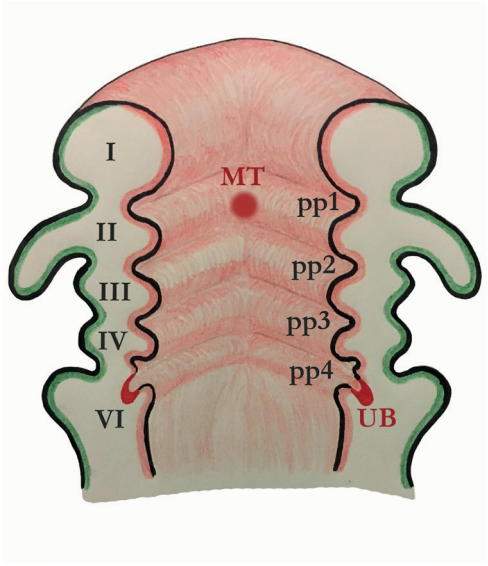

Fig 9. The pharyngeal apparatus from a dorsal view. Roman numerals indicate pharyngeal arches and Arabic numeral indicate pharyngeal pouches (pp). Green color indicates external layer of ectoderm. Corresponding internal layer in red indicates pharyngeal endoderm. The midline thyroid (MT) originates at the level of the second arch. Ultimobranchial bodies (UBs) bud form from the fourth pouches.

mesenchyme end up as muscles and contribute to the formation of pharyngeal vessels (Trainor, Tan, & Tam, 1994).

Patterning of the pharyngeal apparatus is important in providing the cues that determine fates of organ- and tissue-specific cell lineages (Graham, 2003). In this process, NCCs and the gut endoderm act in concert to orchestrate the division of the pharyngeal apparatus into segments with distinct identities and fates (Graham, Okabe, & Quinlan, 2005).

The number of pharyngeal arches differs between species. Mammals generally have fewer arches than lower

vertebrates and in mouse and human the arches are numbered from I to VI (Graham, 2003). The fifth arch forms transiently and soon regresses without leaving traces in the form of any definitive tissues.

Between the arches, pharyngeal pouches are found as outpocketings of the anterior endoderm. The first pair of pouches grows deeper and nearly meets the corresponding clefts, which invaginate from the exterior surface of the embryo. Only a thin membrane that becomes the eardrum will eventually separate them first pharyngeal pouch and cleft. Thus, the first pouch forms the middle ear and the auditory tube, retaining its contact with the definitive pharynx (Grevellec & Tucker, 2010).

The second pouches harbor the palatine tonsils while the endoderm of the third pouches develop into the parathyroid glands and the thymus. In mice, only one pair of parathyroid glands is present, while in humans another parathyroid pair forms from the fourth pouches (Grevellec & Tucker, 2010).

The most distal of the pouches are of special interest in the present work, since they are the source of the ultimobranchial bodies (UBs) (Cordier & Haumont, 1980). The human UBs form as a part of the fifth pouch (Grevellec & Tucker, 2010; Kingsbury, 1915), while the UBs in mice are the only derivatives of the fourth pouches. The name of the ultimobranchial bodies refers to the fact that they come from the most posterior (*ultimo-* means *last*) pharyngeal (or branchial) pouches. The UBs are important in thyroid development since they bring the C cells to the thyroid and in cases where fusion does not occur, no C cells will be present in the thyroid. This is opposed to in non-mammalian species, in which the UBs instead form the ultimobranchial glands that contain C cells, being anatomically separate from the thyroid (Kameda, 2017).

3.2 BUDDING, MIGRATION AND FUSION OF THYROID PRIMORDIA

The entire multistep process of thyroid organogenesis, occurs within one week in the mouse embryo, corresponding to embryonic days (E) 8.5-15.5. Thereafter, further growth and maturation of the follicular thyroid predominates. In humans, thyroid development takes nearly two months, between gestation weeks 3-10 (or E20-70) to accomplish (De Felice & Di Lauro, 2004).

The thyroid primordium develops in the midline, at the level of the second arch. It forms from the ventral part of the pharyngeal endoderm, in close proximity to the aortic sac. In mice, it first appears as a thickening of the endoderm, forming a placode at E8.5, see Figure 10. The placode will further on

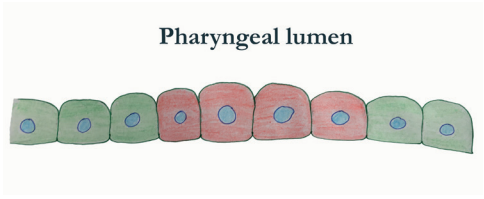

Fig 10. The thyroid placode (red cells) forming in the pharyngeal endoderm around E8.5 in the mouse embryo.

enlarge and bulge out to form the thyroid bud that eventually looses its connection to the pharyngeal endoderm around E10.5, see Figure 11. The bud then descends ventrally of the gut tube and lung buds, and reassumes a close apposition to the aortic sac, after which it expands bilaterally along the third PAA (Fagman, Andersson, & Nilsson, 2006; Rømert & Gauguin, 1973), see Figure 12. The close relation to large vessels has been suggested to function as a guiding track for bilateral growth and symmetrical lobe formation (Alt et al., 2006; Fagman et al., 2006).

As mentioned above, the paired UB buds off from the most posterior pharyngeal pouches, and migrate, surrounded by ectomesenchyme to approach the midline primordium with which it fuses bilaterally around E13.5. The thyroid is thus formed by one medial and two lateral primordia that bring, respectively, follicular cells and C cells to the prospective gland. The fusion process and mechanisms regulating it were addressed in paper II.

3.3 EMBRYONIC GROWTH AND DIFFERENTIATION

After fusion of primordia, the two thyroid lobes gradually enlarge by forming cords radiating from the central parts, represented by the UB remnant, see Figure 13. Recent findings indicate that this developmental stage involves branching morphogenesis under the influence of stromal expression of Fgf10 (Liang et al., 2018). In contrast to the

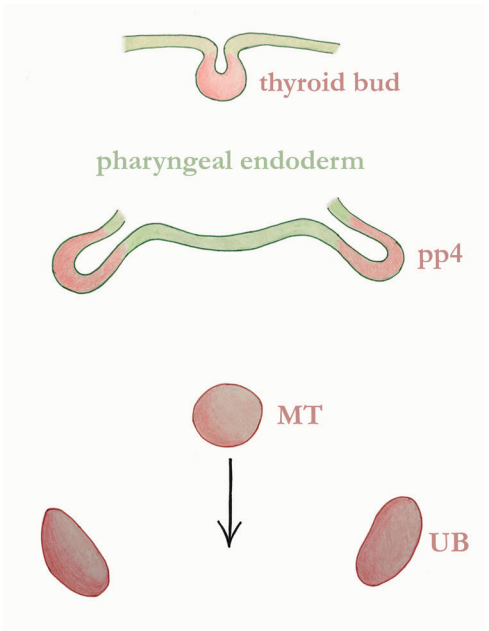

Fig 11. Upper drawings: Thyroid primordia bud from the pharyngeal endoderm in a mouse embryo around E10.5. The thyroid bud is formed at the level shown in Figure 9. The ultimobranchial bodies (UBs) are formed from the fourth pharyngeal pouches (pp).
Lower drawings: Midline thyroid (MT) descends to the level of the UBs after budding, around E11.5 in the mouse embryo.

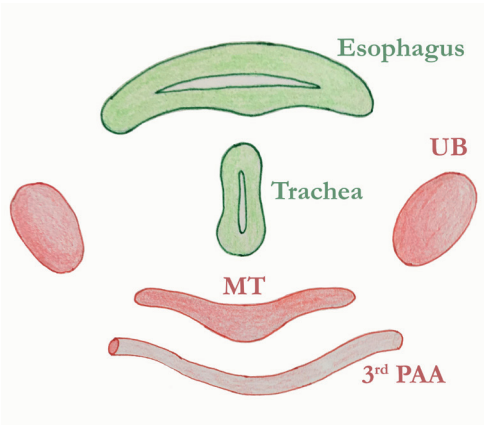

Fig 12. Migration of thyroid primordia. Transverse section through an embryo corresponding to E12.5 in the mouse. The midline thyroid (MT) is growing bilaterally along the same course as the 3rd pharyngeal arch artery (PAA). The relation to the trachea and esophagus are shown.

adult gland, growth of the embryonic thyroid is not dependent on TSH signaling (Postiglione et al., 2002). Figure 13 shows the thyroid early after fusion. Functional differentiation of progenitors is identified by cell-specific expression of Tg and calcitonin, starting around E15.5. This happens in parallel with folliculogenesis, which leads to the formation of many small functional units where hormone synthesis takes place as described above. The regulation of differentiation onset is not completely understood. Trancription factors that are required for this and other stages of thyroid development are discussed in the next section. However, it is unclear how their actions are timely controlled to avoid precocious differentiation. For example, Nkx2-1, Hhex and Pax8 are expressed already as thyroid progenitors assemble in the placode in the pharyngeal floor, but somehow it does not lead to differentiation at such an early stage. The morphogenetic role of vessels in thyroid development has been investigated in different ways. Various extrinsic signals such as Sonic hedgehog influence bilateral growth of the midline thyroid (Henrik Fagman et al., 2006). It is also clear that interactions with endothelial cells and basement membranes are important for the formation of follicles and for their differentiation (Hick et al., 2013; Villacorte et al., 2016).

Keeping thyroid cells in an undifferentiated state is probably important to finish the spatial rearrangements that the thyroid primordia undergo before folliculogenesis and terminal differentiation commence. The significance of a promigratory and undifferentiated phenotype has been discussed not only for embryonic thyroid progenitors, but also for the development of thyroid cancer (Nilsson & Fagman, 2017).

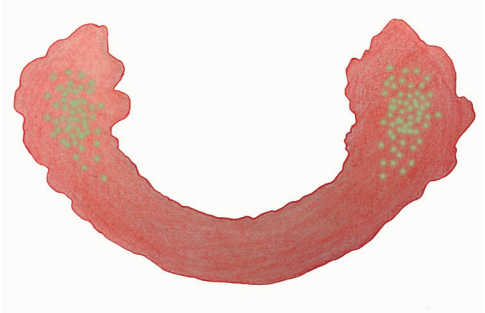

Fig 13. The thyroid gland after fusion with the ultimobranchial bodies (UBs), corresponding to E14.5 in the mouse. UB remnants are visible as scattered cells (green) in the central parts of the lobes that are about to form projections that will grow into cords radiating from the centre of the lobes. The two lobes are connected by the isthmus.

3.4 GENES PARTICIPATING IN THYROID DEVELOPMENT

Much knowledge about thyroid development has been achieved through the studies of both wildtype and genetically engineered mouse embryos (Fernández et al., 2014). The morphogenetic events that lead to the formation of a bilobed gland are well characterized, but the molecular mechanisms that govern different steps of development are not completely understood. Some factors however, are known to be of crucial importance. The combined expression of the four transcription factors Nkx2-1, Pax8, Foxe1 and Hhex

is unique for the earliest formed progenitors of the thyroid follicular lineage (Damante, Tell, & Di Lauro, 2001; Pellizzari et al., 2000). They do not only work alone but also in concert as a regulatory network where the factors interact transcriptionally, which complicates the understanding of their individual roles in thyroid development (Parlato et al., 2004). Each of these factors will be shortly introduced in the following sections, highlighting the relevance for the thesis work. The genes Foxa1, Foxa2 and Sox17 are not part of the transcriptional signature of the thyroid, but they are introduced here, since they play important roles in the work presented in paper I.

3.4.1 Nkx2-1

Formerly known as TTF-1 (thyroid transcription factor 1), Nkx2-1 is a homeobox transcription factor that is expressed in both thyroid primordia, and additionally in the trachea, lungs and forebrain during embryonic development (Lazzaro, Price, de Felice, & Di Lauro, 1991). Soon after thyroid specification in the pharyngeal endoderm, Nkx2-1 can be detected in the midline thyroid and also in the UB epithelium. It is important for the survival of thyroid progenitors, but since the midline thyroid is formed normally in the pharyngeal endoderm even in mice deficient of *Nkx2-1*, it is not necessary for the earliest stages of development (S. Kimura, Ward, & Minoo, 1999). However, these mice are born without a thyroid and die at birth due to lung hypoplasia. Nkx2-1 regulates the expression of thyroid-specific genes such as *Tg* and *TPO* (Fernández et al., 2014) but also stimulates calcitonin production by mature C cells (Suzuki, Katagiri, Ueda, & Tanaka, 2007). Notably, *Nkx2-1* has several domains that seem to be important for differential transcription. This could explain its pleiotropic actions at different stages of development (Silberschmidt et al., 2011). Immunostaining for Nkx2-1 expression was routinely used to identify embryonic thyroid and ultimobranchial cells (papers I and II) and thyroid tumors (paper III). In view of the fact that Nkx2-1 is required for thyroid-UB fusion (Kusakabe, Hoshi, & Kimura, 2006), the thyroid phenotype in mouse embryos haplosufficient for *Nkx2-1* was a subject of study in paper II, devoted to UB and C cell development.

3.4.2 Pax8

The paired box gene *Pax8* is expressed in thyroid cells, only in the follicular lineage. Additionally, Pax8 is of importance for development of a few other organs, most notably the kidneys (Mansouri, Chowdhury, & Gruss, 1998). In *Pax8* null mice, the midline thyroid is formed, but then it regresses and disappears and newborns are severely hypothyroid (Parlato et al., 2004). Since Pax8 is not expressed in the UB epithelium, the UBs develop in *Pax8* knockouts and remain in the normal locations for the missing thyroid lobes, consisting predominantly of calcitonin-producing cells (Mansouri et al., 1998). Pax8 is also important at later stages of development to control differentiation of follicular cells and to prevent apoptosis (Marotta et al., 2014). In paper II, staining for Pax8 was used to distinguish cells originating from the mid-

line thyroid from cells originating from the UBs, and the UB phenotype was studied in *Pax8* deficient embryos.

3.4.3 Foxe1 and Hhex

Foxe1 belongs to the forkhead domain transcription factor family and was previously known as thyroid transcription factor 2, or TTF-2 (Civitareale, Lonigro, Sinclair, & Di Lauro, 1989; Fernández et al., 2014). It is widely expressed in pharyngeal endoderm, but not in the second, third or fourth pharyngeal pouches (Dathan, Parlato, Rosica, De Felice, & Di Lauro, 2002). Mice deficient of *Foxe1* die shortly after birth by failure to thrive due to a cleft palate, but they also have a severe thyroid phenotype comprising athyreosis, alternatively a remaining, small thyroid remnant located under the tongue (De Felice et al., 1998). It is suggested that Foxe1 downstream of Nkx2-1 and Pax8 drives migration of the thyroid primordium after budding is completed (Parlato et al., 2004). *FOXE1* is also a susceptibility gene in thyroid cancer (Jones et al., 2012).

The hematopoietically expressed homeobox gene *Hhex* is expressed in the embryonic thyroid but also in other foregut derivatives as liver, lung and thymus (Bedford, Ashworth, Enver, & Wiedemann, 1993; Bogue, Ganea, Sturm, Ianucci, & Jacobs, 2000). In *Hhex*$^{-/-}$ mice, the midline thyroid is primarily formed and Nkx2-1, Pax8 and Foxe1 are initially normally expressed. However, these transcription factors are soon thereafter downregulated, suggesting an important role for Hhex in maintaining their expression (Martinez Barbera et al., 2000; Parlato et al., 2004). Foxe1 and Hhex were not specifically studied in this thesis and will not be further discussed.

3.4.4 Foxa1 and Foxa2

There are three members of the forkhead box A gene family; *Foxa1, Foxa2* and *Foxa3*. The first two of these factors cooperate in embryonic development and are partly redundant (Kaestner, 2010). Foxa2 is ubiquitously expressed in the pharyngeal endoderm but is downregulated in thyroid progenitors before the thyroid bud is formed (Fagman et al., 2011). Both Foxa1 and Foxa2 act as pioneer factors, which means that they regulate chromatin organization and thereby make the distinct parts of the genome available for the binding of other transcription factors (Kaestner, 2010). Foxa2 has been shown to regulate the *calcitonin* promoter in C cells (Viney et al., 2004). The *Foxa* genes are crucially important also for the development of other organs that derive from foregut endoderm, such as lung and liver (Costa, Kalinichenko, Holterman, & Wang, 2003; Hannenhalli & Kaestner, 2009), which makes the thyroid an interesting exception. In paper I, the expression pattern of Foxa1 and Foxa2 was investigated in the UB epithelium and embryonic C cells in mouse, and in tumor samples of human medullary thyroid cancer.

3.4.5 Sox17

There are 20 genes belonging to the Sox family comprising SRY (sex-deter-

mining region of the Y chromosome) –related HMG (high mobility group) –containing box genes (Kiefer Julie, 2007). Sox17 is important for the specification and formation definitive endoderm (Séguin, Draper, Nagy, & Rossant, 2008). It is expressed in the entire gut tube of early mouse embryos, but is down-regulated in the foregut endoderm around E8.5 (Kanai-Azuma et al., 2002). In paper I, tracing the progeny of endoderm cells that previously expressed Sox17 (Engert, Liao, Burtscher, & Lickert, 2009) was used to investigate their contribution to progenitor cells of the thyroid and UB.

3.5 THYROID DEVELOPMENTAL DEFECTS

In humans, significant disturbances in thyroid development cause a variety of clinical conditions in which thyroid function is impaired or even lost. Congenital hypothyroidism (CH), is a fairly common developmental defect with an incidence around 1/3500 newborns (Toublanc, 1992). CH can be caused by dyshormonogenesis (the inability to produce thyroid hormones), accounting for approximately 15 % of cases, or thyroid dysgenesis, in which impaired organogenesis lead to athyreosis (no thyroid is found), hypoplasia (the size of the thyroid is reduced) or ectopia (thyroid tissue present at other locations than normal), accounting for the remaining 85 % of cases (De Felice & Di Lauro, 2004).

Before birth, maternal thyroid hormones can compensate for the lack of endogenous production in the embryo, but children that are born with insufficient ability to produce thyroid hormones will develop symptoms ranging from mild hypothyroidism to cretinism, characterized by dwarfism and mental retardation (Vulsma, Gons, & de Vijlder, 1989). Most cases of CH are sporadic. Notably, dysgenesis associated with mutations in known thyroid developmental genes as *NKX2-1, PAX8* and *FOXE1* comprise only a minority of cases (De Felice & Di Lauro, 2004; Macchia et al., 1998; Nettore, Cacace, De Fusco, Colao, & Macchia, 2013). Familial clustering exists, and abnormal thyroid function is more common in first-degree relatives to children with CH, than in controls (Sindhuja, Dayal, Sodhi, Sachdeva, & Bhattacharya, 2016), although thyroid dysgenesis is often discordant in monozygotic twins (Perry et al., 2002).

Occasionally, thyroid dysgenesis is associated with other developmental disorders, most notably cardiac malformations (Olivieri et al., 2002). *NKX2-1* haploinsufficiency has been identified in some patients with different degrees of CH together with pulmonary dysfunction, choreoathetosis, and muscular hypotonia (Krude et al., 2002). There are a few cases in which athyreosis is seen in combination with cleft palate and spiky hair, with or without bilateral choanal atresia, due to mutations in *FOXE1* (Castanet & Polak, 2010). Also, thyroid dysgenesis is common in patients with DiGeorge syndrome,

caused by a 22q11 deletion (Stagi et al., 2009). These patients typically suffer from defective development of the cardiac outflow tract and PAAs, i.e. closely related to the thyroid developmental pathway.

Thanks to neonatal screening programs, children with CH are generally diagnosed early and subjected to hormone substitution therapy, which rescues them from severe symptoms such as impaired neurological development (Seo, Yoon, So, Lee, & Hwang, 2017).

3.6 THE ORIGIN OF C CELLS

In mammals, C cells become integrated with the thyroid gland instead of forming ultimobranchial glands as in lower vertebrates. Thyroid C cells belong to the neuroendocrine series and were originally considered to be of neuroectodermal origin. However, the classification of neuroendocrine organs and tissues has changed over time according to that more advanced techniques have helped to a better understanding of the characteristics and origins of different cell types. This chapter provides a background to the rationale of investigations in paper I.

3.6.1 The neuroendocrine system and the APUD cells

The interactions between neuronal and endocrine tissues were recognized early, and the understanding of the regulatory systems of the human body emerged step by step. In 1938, Friedrich Feyrter proposed the existence of a new endocrine system with a widespread location in many organs, diffusely spread in the tissues and with important connections to the nervous system (Modlin, Champaneria, Bornschein, & Kidd, 2006). He also suggested that these endocrine cells originated from the nervous system. The concept of the neuroendocrine system was further supported by the works of others (Pagès, 1957; Sunder-Plassmann, 1939) and included thyroid C cells as well as endocrine cells from the pituitary gland, adrenal glands, stomach, intestine and pancreas (Pearse, 1969).

During the 60s and the 70s the neuroendocrine cells were found to have, similar to neuronal cells, the capacity of amine precursor uptake and decarboxylation, and the acronym APUD was suggested as a name of this group of cells. The APUD cells were thought to have a common NC origin (Pearse, 1969), but it was later shown that most of the cells included in the neuroendocrine system were in fact stemming from endodermal tissues (Andrew, Kramer, & Rawdon, 1982, 1983; Fontaine & Le Douarin, 1977; Le Douarin & Teillet, 1973).

Today, the concept of the neuroendocrine system has changed and the misleading APUD acronym is more or less abandoned. Rather than focusing on a common embryonic origin, the criteria for neuroendocrine cells now rely on the molecular phenotype, such as the production of neuropeptides and the presence of secretory granules in cells that do not signal via synapses (Day & Salzet, 2002).

3.6.2 Tracing neural crest cells using quail- chick chimeras

Before genetic tracing techniques were available, there were other ways of investigating the movement of cells during embryonic development. The use of avian chimeras allowed for the tracing of cells with certain nuclear features in a xenograft model (Douarin Nicole & Dieterlen-Lièvre, 2013). This studied the fate of NCCs by surgically replacing a defined portion of the NC in chick embryos with the corresponding NC segment obtained from quail embryos. The embryos were then left to develop and later on examined to observe where the quail cells ended up. Notably, chick and quail cells can be distinguished from each other on the basis of different nuclear characteristics and these experiments showed that quail cells migrated to invade a multitude of embryonic tissues. Quail-chick chimeras were thus instrumental in characterizing the fate of NCC which revolutionized our understanding of vertebrate development (Douarin Nicole & Dieterlen-Lièvre, 2013). One of the conclusions drawn from this model was that NCCs populate the ultimobranchial gland in the chick embryos. The same cells were also found to have immunoreactivity for calcitonin, i.e. they were identified as C cells (Polak, Pearse, Le Lievre, Fontaine, & Le Douarin, 1974).

3.6.3 Controversies on the embryonic origin of thyroid C cells

Ever since, the prevailing concept in textbooks of embryology has been that C cells are derived from NC also in mammals, despite the lack of evidence supporting this idea. As mentioned, in all vertebrates except mammals, the UB does not fuse with the midline thyroid, but forms the ultimobranchial gland. Still, the data from experiments in avian species on the embryonic development of C cells were extrapolated and assumed to hold true also for mammals.

With the development of better tracing techniques and refined imaging possibilities, doubts have been raised regarding the NC origin of thyroid C cells. In 2007, it was shown that tracing of cells derived from the NC failed to include cells that ended up in the mouse thyroid, other than stromal cells (Kameda, Nishimaki, Chisaka, Iseki, & Sucov, 2007). In paper I, lineage tracing of endoderm progeny was used to investigate the embryonic origin of mouse C cells.

”Growth for the sake of growth is the ideology of the cancer cell”

Edward Abbey
(1927–1989)

4 Thyroid cancer

THYROID CANCER

Malignant tumors of the thyroid represent the most common form of endocrine cancer and constitute a wide spectrum of phenotypes from tumors with excellent prognosis to one of the most aggressive tumors in humans (American Cancer Society, 2017; Cabanillas, McFadden, & Durante, 2016). Most tumors arising belong to the group of differentiated thyroid cancers (DTC), all of which derive from mutant follicular epithelial cells. DTC can be further divided into subgroups with papillary thyroid carcinoma (PTC), and follicular thyroid carcinoma (FTC), distinguished by both the histological appearance and clinical characteristics. These two subtypes are also the most prevalent. Poorly differentiated thyroid cancers (PDTC) usually exhibit some features of the originating follicular cells, but have a more aggressive course than DTC, due to dedifferentiation of the tumor cells. Anaplastic thyroid cancer (ATC) is a completely undifferentiated and highly malignant tumor. It may develop from DTC/PDTC or arise *de novo*, possibly from a stem cell population (Cabanillas et al., 2016).
Medullary thyroid carcinoma (MTC) originates from C cells and thus represents a different entity. MTC tumors are neuroendocrine due to their cell origin and expression of calcitonin (DeLellis, 2011).
Papers III and IV investigate a mouse model for PTC, which warrants a closer description of this thyroid cancer type in the following. MTC will also be briefly described since it forms a background to part of the studies in paper I. But first a few words on how thyroid cancer patients are managed clinically.

4.1 CLINICAL PRESENTATION AND MANAGEMENT

When a patient presents with thyroid enlargement, the possibility of a thyroid cancer has to be considered. Benign thyroid nodules are common and among these patients, it is important to sort out those where no intervention is needed, from those who have a thyroid cancer (Haugen et al., 2016). Since the thyroid is located close to the trachea,

a patient with thyroid enlargement can experience symptoms related to compression, such as difficulties in breathing or swallowing. Other symptoms may include neck pain, swollen lymph nodes, hoarseness or, uncommonly, symptoms related to deranged hormone levels.

In 2016, the American Thyroid Association (ATA) published updated guidelines for the management of thyroid nodules and DTC in adult patients (Haugen et al., 2016). There are also guidelines for the management of MTC that were published by ATA in 2015 (Wells et al., 2015). In this section the guidelines will be briefly reviewed. In Sweden, there is a national program for the management of thyroid cancers and this program adheres to the ATA guidelines (Regionalt cancercentrum väst, 2017).

If a thyroid tumor is suspected, a clinical examination and ultrasound-guided fine-needle aspiration (FNA) for cytological evaluation are performed and they are the cornerstones of the preoperative, diagnostic procedure. Analysis of serum TSH is performed to evaluate the functional state of the thyroid and as prognostic factor, since high levels of TSH indicate a higher risk of malignancy (Boelaert et al., 2006; Polyzos et al., 2008). Serum levels of calcitonin could be determined to find indication of MTC (Elisei et al., 2004; Wells et al., 2015).

The result of the cytological examination is reported according to the Bethesda system (Crippa & Mazzucchelli, 2010), which gives an estimation of the risk of malignant disease. In the presence of malignant cells or suspected malignant disease, patients are referred to surgical treatement by thyroidectomy (see section 4.7 for more information on treatment adjuvant to surgery).

4.2 PAPILLARY THYROID CARCINOMA (PTC)

4.2.1 Epidemiology and etiology

PTC is the most common form of thyroid cancer and accounts for around 70 % of all cases. It occurs three times more often in women than in men. Thyroid cancers overall has shown a markedly rising increase over the last decades, mostly due to an increase in the incidence of PTC (Kilfoy et al., 2009; La Vecchia et al., 2015). Reasons for this have been a matter of debate. Even if improved diagnostic tools is likely to explain most of it, there is still an increased number of cancer patients also diagnosed with large tumors that cannot be explained by known factors (Kitahara & Sosa, 2016).

One important environmental factor that leads to higher risk of PTC is the exposure of ionizing radiation, due to both medical x-ray examinations and incidental ingestion of radioactive iodine after nuclear plant accidents. The Chernobyl catastrophe is an example of how exposure of radioactive isotopes being spread all over Eastern Europe, in particular Ukraine and Belarus, caused an alarming increase in the in-

cidence of childhood PTC (LiVolsi et al., 2011; Williams, 2006). Other less well characterized risk factors of developing thyroid cancer include reproductive factors (Rahbari, Zhang, & Kebebew, 2010), dietary factors (Z.-T. Liu & Lin, 2014), smoking (Cho & Kim, 2014) and diabetes (Yeo et al., 2014).

4.2.2 Histology and subtypes of PTC

The microscopic diagnosis of PTC is based on the presence of a papillary growth pattern and typical nuclear features, including change of nuclear size, pseudoinclusions, nuclear grooves, and ground glass nuclei in the tumor cells (Albores-Saavedra, Altamirano-Dimas, Alcorta-Anguizola, & Smith, 1971; Lloyd, Osamura, Klöppel, & Rosai, 2017). The last phenomenon has also been described as clear nuclei or orphan Annie nuclei (Dominguez-Malagon Hugo, Szymanski-Gomez Juan, & Gaytan-Garcia Silvia, 2006). The predominant growth pattern is characteristic with papillary formations that can form straight or branching cords. Sometimes there are also other growth patterns in combination, such as follicular or trabecular arrangements (Lloyd et al., 2017).

Apart from the classical PTC there are 14 variants listed by WHO and their names refer to typical features in each of them regarding size (microcarcinoma), tumor cell shape (tall cell, columnar cell, hobnail, oncocytic, spindle cell and clear cell variants), secondary growth pattern (follicular, diffuse-sclerosing, cribriform-morular, and solid/trabecular variant) or the stromal contribution (encapsulated, PTC with fibromatosis/fasciitis-like stroma, Warthin-like variants) (Lloyd et al., 2017).

PTC subtyping is of prognostic value. However, due to lack of suitable models for experimental investigation, the basic mechanisms behind the differences in tumor cell behavior of PTC subtypes, conferring accelerated growth, local invasiveness and metastasizing capacity, are largely unknown. Paper III presents a novel PTC mouse model that might be instrumental on these aspects.

4.3 MEDULLARY THYROID CARCINOMA (MTC)

4.3.1 Epidemiology and etiology

MTC derives from C cells and accounts for around 5-10 % of all thyroid cancers. Most cases are sporadic, but approximately 25 % of cases are hereditary, either as familial MTC (FMTC) in isolation, or as a part of the multiple endocrine neoplasia (MEN) syndrome type 2A or 2B (Leboulleux, Baudin, Travagli, & Schlumberger, 2004). In MEN 2A, MTC is associated with phaeocromocytoma and hyperparathyroidism, while in MEN 2B, MTC occurs together with phaeochromocytoma, ganglioneuromatosis and different muscular and skeletal malformations.

MTC is mostly located at the junction between the two upper thirds of the gland. In 20 % of cases, distant metastases are present already at diagnosis,

most often in the liver, lungs or bones (Leboulleux, Baudin, Travagli, & Schlumberger, 2004).

4.4 THE MAPK/ERK SIGNALING PATHWAY

Several oncogenic mutations are involved in the development of thyroid cancer. They mostly affect the mitogen-activated protein kinase (MAPK) pathway, which motivates a closer presentation of its key signaling molecules. Within this pathway there are several cascades with different mediators (Pritchard & Hayward, 2013). This description will focus on the cascade that ends with the extracellular signal-regulated kinase (ERK) and this pathway is also called the MAPK/ERK pathway. These are the players in the pathway:

- RTK
- Ras
- Raf
- MEK
- ERK

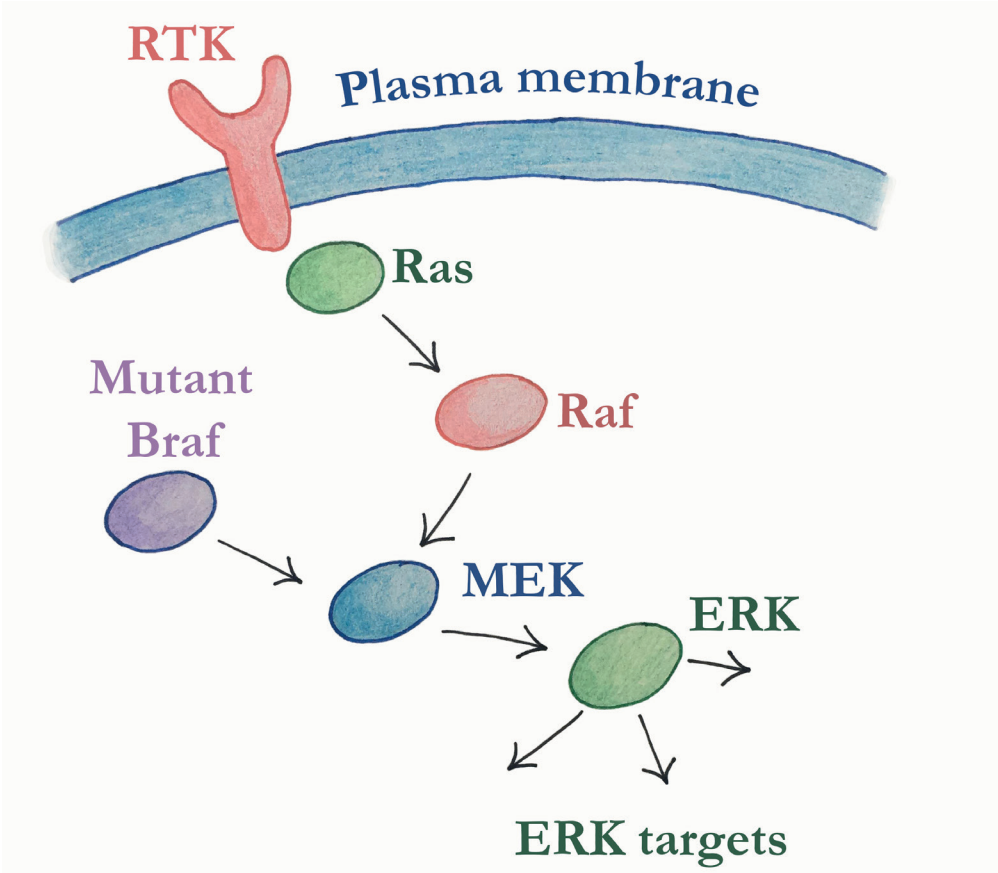

Fig 14. Schematic illustration of the MAPK/ERK pathway. A receptor tyrosine kinase (RTK) is shown, located at the basal plasma membrane of a follicular cell. When a ligand binds, the RTK will activate Ras, which in turn activates Raf - MEK - ERK - various targets. Mutant Braf is constitutively active and can activate downstream targets even in absence of Ras signaling.

4.4.1 Receptor tyrosine kinase

Receptor tyrosine kinases (RTKs) are membrane-spanning proteins located at the cell surface. Commonly they form dimers after binding of their ligand which causes autophosphorylation required for activation of the receptor (McKay & Morrison, 2007). The activated receptor can then elicit different cascades of intracellular signaling to control cell activity such as cell differentiation, proliferation and migration (Schlessinger, 2000). I will here focus only on elements that eventually activates ERK.

4.4.2 Ras

After RTK, the next step in the MAPK pathway is the small G protein Ras, which can exist in a guanosine diphosphate(GDP)-bound, inactive or a guanosine triphosphate(GTP)-bound, active state (Bar-Sagi & Hall, 2000). RTK activation stimulates the exchange of GDP for GTP by guanine nucleotide exchange factors (GEFs) to activate Ras. The GTP of the activated Ras will then be hydrolysed to GDP by GTPase activating proteins (GAPs) and the protein will return to its inactive state (Lowy & Willumsen, 1993).

There are three Ras genes (H-ras, N-ras, and K-ras) encoding four proteins that play slightly different roles in cellular signaling. The different isoforms can undergo oncogenic activation and they were discovered due to their involvement in different cancers. Oncogenic activation is related to mutations that keep the protein in an active state, thus the pathway is constitutively activated, causing aberrant signaling (Castellano & Santos, 2011).

4.4.2 Raf

The Raf family includes three serine/threonine kinases and follows Ras activation in the MAPK pathway. The *Raf* gene was first discovered through the cloning of a viral oncogenic that induces rapidly growing fibrosarcoma in mice and this oncogene was called *v-raf*, which later was found to have a homologue, *c-raf*, in eukaryotic cells (Rapp et al., 1983; Schreck & Rapp, 2006). Additional homologues were found in mammals and named *a-raf* and *b-raf* (Daum, Eisenmann-Tappe, Fries, Troppmair, & Rapp, 1994).

All the three Raf kinases possess three conserved regions, CR1, CR2, and CR3 that are important for their function and regulation. CR1 is a domain to which Ras binds and activates Raf. CR2 can bind an inhibitory protein to negatively regulate the kinase activity. Lastly, CR3 is the protein kinase domain that phosphorylates the downstream kinase MEK (Lavoie & Therrien, 2015; Zebisch & Troppmair, 2006).

4.4.3 MEK and ERK

There is substantial cross talk between different intracellular signaling pathways, but the only known downstream target for the Raf kinases is MEK (MAPK/ERK kinase) which in turn only have one downstream target in ERK (extracellular regulated kinase). Activation of ERK by MEK facilitates its transport into the nucleus where it

regulates transcription factors involved in processes such as cell proliferation and differentiation (Plotnikov, Zehorai, Procaccia, & Seger, 2011; Yang, Sharrocks, & Whitmarsh, 2013).

To summarize, the MAPK pathway receives extracellular signals activating an intracellular signaling cascade that alters transcription of target genes. Modification and regulation of the signal is possible at several levels. Because of the crosstalk between the MAPK and other signaling pathways, there are many outcomes possible through a limited number of kinases involved.

4.5 MUTATIONS OF THE MAPK PATHWAY

Genetic alterations conferring constitutive activation of the MAPK pathway are common oncogenic drivers in various cancers. The most prevalent mutations leading to cancer development involves *RAS* and *RAF*, leading to uncontrolled tumor cell proliferation and often also dedifferentiation (Karnoub & Weinberg, 2008; Pylayeva-Gupta, Grabocka, & Bar-Sagi, 2011). Interestingly, mutations of Ras and Raf are mutually exclusively and are never encountered in the same tumor cell (Cisowski, Sayin, Liu, Karlsson, & Bergö, 2015; Rajagopalan et al., 2002).

4.5.1 Braf mutations

Mutated *BRAF* occurs in 40-45 % of PTC cases (R. Ciampi & Nikiforov, 2005; Cohen et al., 2003; E. T. Kimura et al., 2003). Besides being involved in thyroid cancer, mutant *BRAF* is often found in melanomas and colorectal cancer (Rajagopalan et al., 2002). Most of the known oncogenic *BRAF* mutations involve alterations at or close to the sequence that encodes for the activation site of the kinase (Davies et al., 2002). The most prevalent mutation is a substitution of glutamic acid for valine at position 600 ($Braf^{V600E}$), by which the encoded kinase constantly possesses its activated conformation (Ikenoue et al., 2004).

The effects on cell behavior as a response to Raf signaling differs due to different levels of kinase activity. This means that a higher level of kinase activity does not necessarily lead to increased cell proliferation. Instead, increased Raf activity due to mutation/overexpression has in some cases been shown to induce cell cycle arrest (Woods et al., 1997). This highlights the complex nature of the MAPK signaling pathways regulating cell growth responses.

In mouse thyroid, conditional expression of $Braf^{V600E}$ initiates tumorigenesis and also dedifferentiation of neoplastic thyroid cells (Knauf et al., 2005). It downregulates the genes for NIS, Tg and TPO, and therefore impairs normal thyroid function severely. A similar model of thyroid cancer development forms the basis of the studies in papers III and IV.

4.5.2 RAS mutations

Mutations of all three isoforms *HRAS, KRAS* and *NRAS*, are all found in thyroid cancers (Howell Gina, Hodak Steven, & Yip, 2013; Suarez et al., 1990).

RAS mutations are most prevalent in FTC and are also reported to occur in the follicular variant of PTC (Zhu, Gandhi, Nikiforova, Fischer, & Nikiforov, 2003). This indicates that mutant Ras acting upstream of Braf confers a different tumor phenotype, which is interesting. Besides activating the MAPK pathway, participation of Ras signaling in other pathways, most notably PI3K/AKT, may explain the differential oncogenic response (Z. Liu et al., 2008).

4.5.3 RET mutations

RET is a proto-oncogene that was found in 1985 and the name comes from Rearranged upon Transfection (Takahashi, Ritz, & Cooper, 1985). *RET* encodes an RTK and is involved in the MAPK pathway but, like RAS, it also effects the PI3K pathway (Besset, Scott, & Ibáñez, 2000; Hayashi et al., 2000). There are two types of genetic alterations that make *RET* oncogenic. First, there are point mutations that occur both as sporadic mutations and germ line mutations. This type of alteration is typically found in MTC and the MEN2 syndrome. Second, the tyrosine kinase domain may fuse with another gene, so-called *RET/PTC* rearrangements, that encodes an intracellular oncogenic fusion protein. *RET/PTC* is found in 20-40 % of PTC cases (Raffaele Ciampi & Nikiforov, 2007; Romei, Ciampi, & Elisei, 2016).

4.6 ONCOGENE-INDUCED SENESCENCE AND THE ROLE OF TSH

Cell cycle arrest in response to oncogene activity is called oncogene-induced senescence (OIS) and has entered the limelight in recent years. Similar to cellular senescence well-known as a limit for the propagation of cells in cell culture, OIS leads to cell cycle arrest in the G1 phase, accompanied by slowdown of cellular metabolism and also morphological changes (Reddy & Li, 2011). Cells that are senescent typically become flat and elongated. They also express tumor suppressor proteins such as p16 and p53, which are important in mediating cell cycle arrest (Moulana, Priyani, de Silva, & Dassanayake, 2018). Conceivably, OIS prevents cells from propagating DNA damage to their progeny, and is therefore important in protecting the organism from cells that might malignify and give rise to cancer. However, OIS can be overcome by different mechanisms. In the thyroid, increased TSH signaling can overcome OIS and lead to tumor development (Zou et al., 2015). In a hypothyroid state, the pituitary gland compensates by increased levels of TSH. Such a state sometimes occurrs in thyroid tumors, since mutant Braf causes dedifferentiation and loss of functioning epithelium. Also, mutant Braf upregulates the TSH receptor on the follicular cells and amplifies the effect of TSH signaling in thyroid cells (Moulana et al., 2018).

4.7 TARGETED TREATMENT STRATEGIES

The standard treatment for PTC is surgery, often combined with radioactive iodine (RAI) therapy and occasionally

external beam therapy (Haugen et al., 2016). The principle behind RAI is that follicular cells are the only cells in the body with a possibility to concentrate iodine. When a patient receives treatment with RAI, the effect is therefore restricted to the thyroid (or metastases from thyroid cancer), sparing other tissues. RAI has been used for the treatment of hyperthyroidism and thyroid cancer since the early 40s (Daniels, 2012; Gold, 1951). The isotope used in clinical practice is ^{131}I and one prerequisite for successful RAI therapy is that the tumor cells express NIS, so that radioiodine can be concentrated intracellularly or accumulated in preserved follicular lumens to achieve a sufficient radiation dose and therapeutic effect. An obvious problem is dedifferentiation, prevalent in many PTC tumors, in which mutant Braf down-regulates the expression of NIS, making tumor cells refractory to RAI treatment (Schlumberger, Lacroix, Russo, Filetti, & Bidart, 2007).

Advances in the understanding of oncogenic drivers in cancer have led to the development of drugs that specifically inhibits various kinases involved in the MAPK and other signaling pathways. Several tyrosine kinase inhibitors (TKIs), as vandetanib, cabozantinib, sorafenib, and lenvatinib have been approved for adjuvant treatment of advanced thyroid cancer (Valerio et al., 2017). This type of treatment holds promise in that it affects the pathways that are directly involved in tumor development. However, they also have severe side effects that limit their use and only patients with advanced cancer are subjected to this kind of therapy (Mohammed & El-Shentenawy, 2014). Drug resistance is also a limiting factor. Vemurafenib (PLX4032) is a TKI that is active against mutant Braf (Joseph et al., 2010). It has been approved for the use in patients with melanoma (Kim et al., 2014) and effects the dimerization of BRAF, impairing its activity (Karoulia, Gavathiotis, & Poulikakos, 2017). In paper IV, vemurafenib was tested for inhibition of early tumorigenesis in the Braf^{V600E} mouse model of PTC.

The outcome for thyroid cancer patients with adequate treatment is very good, and the 10-year relative survival is more than 90 % for all histological types. PTC has the best prognosis with a 10-year relative survival of 98 %, while the corresponding numbers for FTC is 92 %, for MTC 80 %, and for anaplastic cancer 13 % (Gilliland, Hunt, Morris, & Key, 1997).

4.8 CANCER DEVELOPMENT

Tumors elicited by one and the same oncogenic mutation show a phenotypic heterogeneity that reflects the complexity not only of the genetic events that lead to cancer, but also of the originating cells that are primarily mutated (Visvader, 2011). The development of cancer is often described as a chain of events that leads to transformation of a normal cell. Such a cell is called a tumor-initiating cell (TIC) and should be distinguished from cancer stem cells

(CSCs), which function as tumor-propagating cells. TIC and CSC are sometimes used synonymously, which may be confusing (Rycaj & Tang, 2015; Visvader, 2011). The concept of CSCs is still evolving and their origin and characteristics are uncertain (Hanahan & Weinberg, 2011). However, the nature of TICs is of fundamental interest to understanding tumor heterogeneity. For example the differentiation status of a TIC might influence its progress in tumor development and explain differences in tumor phenotypes arising from identical oncogenic mutations. In skin cancer, there are studies that argue for a stem cell origin, as well as for an origin in mature cells that dedifferentiate and initiate tumor development (Köhler et al., 2017; Malanchi et al., 2008).

As previously mentioned the $Braf^{V600E}$ mutation is involved in tumor-initiation in several tissues, such as the colon and the skin. These tumors do not progress to malignant disease unless other genetic alterations appear. In contrast, $Braf^{V600E}$ has shown the ability to alone transform follicular cells and cause them to progress to PTC (Knauf et al., 2005). This implies different susceptibility of the tumor-initiating cells between different organs and tissues. The mechanism behind this difference is poorly understood but may be related to the inborn growth regulation that differs much among cell types. In both colon and skin, there is a high turnover of the epithelial cells and homeostasis is achieved through the presence of tissue stem cells that constantly renew the epithelium to compensate for the continuous loss of cells through shedding at the epithelial surface (Yan & Owens, 2008). This is in sharp contrast to thyroid follicular cells, which normally replicates around five times during the normal human life-span (Coclet et al., 2008). A high turnover means that cells have less time to accumulate additional mutations, which means that cells in for example the skin and intestine are protected by their short lifespan.

Another level of tumor heterogeneity concerns the phenotypic variations of PTC, which range from microcarcinoma to advanced and invasive tumors, with different prognostic outcome. Notably, among the majority of PTC cases that carry the $Braf^{V600E}$ mutation, the whole spectrum of tumor phenotypes exist of which only a minority progresses to advanced disease with mortality risk. Present conditional mouse PTC models, in which mutant Braf is globally and synchronously expressed in all thyroid cells are invalid for investigation of a possible heterogenic response of single tumor cells within the normal thyroid microenvironment. In papers III and IV, a new mouse model of sporadic PTC was developed and characterized on the basis of spontaneous Cre-induced $Braf^{V600E}$ activation. This model allowed for the detailed study of tumor initiation and development.

”Never be so focused on what you are looking for that you overlook the thing you actually find”

Ann Patchett
(b. 1963)

5 Aims of the thesis

AIMS OF THE THESIS

The aims of the thesis were to investigate:

- a possible endodermal embryonic origin of thyroid C cells and its implications for the tumor phenotype in medullary thyroid carcinoma
- epithelial and migrating properties of embryonic C cells before, during and after fusion of the ultimobranchial bodies with the embryonic thyroid
- *Braf*^{V600E}-induced tumor development in the natural microenvironment by generating a mouse model of sporadic papillary thyroid cancer (PTC)
- tumor initiation and clonal development of thyroid microcarcinomas from single Braf mutant follicular cells
- effect of a Braf ^{V600E} inhibitor on early tumorigenesis in the sporadic PTC model

”A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales”

Marie Curie
(1867–1934)

6 Methodology

METHODOLOGY

Different techniques to intervene with, trace, and image cells form the cornerstone of this thesis. Each of them presents with different advantages and disadvantages. In this chapter, the most important techniques used in the four papers will be explained and discussed.

6.1 GENETIC ENGINEERING

Creating genetic alterations in cell culture or laboratory animals is a way to achieve important information about the functions of specific genes (Bouabe & Okkenhaug, 2013). To knockout a gene and then simply observe what happens is an appealing strategy, but it has turned out that it is not an as straightforward approach as one could wish. A single genes is involved in so many functions in an organism. Therefore, deleting one gene often causes such profound effects that it is difficult and sometimes impossible to discern its functions in a given tissue context. Also, some genes are crucial during embryonic development and their absence could therefore be embryonically lethal, precluding the study of the function later on in development or in adult animals (Doyle, McGarry, Lee, & Lee, 2012). Yet another complicating factor is that many genes cooperate with other genes so that the effect of a single gene knockout may be related to altered functions of partner gene(s). Sometimes there is redundancy between two or more genes, so that deletion of either one of the genes causes little or no effect; knockout of both is required to uncover a dysregulated mechanism.

6.1.1 Cre/loxP-system

The discovery of the bacterial recombinase Cre (the name is short for causes recombination) has provided important new tools for genetical engineering. Cre is an enzyme that recognizes specific nucleotide sequences called loxP (from locus of x-over of bacteriophage P1) sites (Sternberg & Hamilton, 1981). The loxP site is a DNA sequence of 34 basepairs with a centre of 8 basepairs and two palindromic 13 basepair sequences on each side of the centre. This sequence does not occur in the natural mouse genome, but it can be inserted on each side of a specific

gene and this gene is then said to be floxed (flanked by loxP). Cre is able to cause recombination between two loxP sites and the result can be deletion or inversion of the gene depending on the direction of the loxP sites (Smedley, Salimova, & Rosenthal, 2011). See Figure 15 for a schematic representation of the Cre/loxP system.

6.1.2 Spatial control of Cre expression

The application of the Cre/loxP-system in transgenic mice has opened new possibilities in tailoring mouse models of disease or for the understanding of normal gene function in a given organ, tissue or cell type (Orban, Chui, & Marth, 1992). Typically a tissue-specific promoter controls the expression of Cre. Thus, the expression of Cre can be restricted, allowing for spatial control of the genetic event caused by Cre to only the cells that normally express a gene controlled by the same promoter. Other cells will not express Cre and a floxed gene in these cells remains unaffected. To date, there is a plethora of different Cre drivers, which enables studies of numerous variants designed for conditional manipulation of the targeted genes of interest (Nagy, Mar, & Watts, 2009).

6.1.3 Temporal control of Cre expression

Many genes are crucial for embryonic development and their early deletion could make it impossible to investigate their role in adulthood. To achieve temporal control of Cre-mediated recombination, some variants have been designed in which Cre is coupled to an additional element that restricts it to the cytoplasm. Cre-ER^{T2} is a variant in which a modified estrogen receptor is coupled to Cre and prevents it from entering the cell nucleus to perform recombination (Feil, Wagner, Metzger, & Chambon, 1997). Upon binding to the receptor by its ligand tamoxifen, Cre will be allowed to enter the nucleus. Thanks to this, the effect of Cre-dependent recombination can be temporally controlled and prevented until administration of the inducing agent.

6.1.4 Knock-outs and knock-ins

The Cre/loxP-system is a very convenient tool since it only requires two principal elements to create a genetic alteration. The system can produce both conditional deletion (knockout) and conditional expression (knockin) of specific genes, see example in figure 16. To accomplish this, a gene is floxed, or a floxed stop-cassette is placed upstream of a gene (that might be endogenous or inserted), respectively. A floxed stop-cassette prevents the expression of the gene. Cre-mediated recombination then removes the floxed gene (knock-out) or the stop-cassette (knock-in). In the latter case removal of the stop-cassette makes the gene available for transcription. This allows for the study of loss- or gain-of-function of the gene after recombination (Doyle et al., 2012).

6.1.5 Problems in using Cre/loxP

Several unexpected findings have shown that there are important aspects to consider when using the Cre/loxP-system and interpreting results obtained from it. For example, animals expressing Cre might have an altered phenotype even in the absence of recombination (Forni et al., 2006; Loonstra et al., 2001). Another issue concerns the fidelity of spatial and temporal control elements. Unexpected Cre expression in off-target tissues has been reported and leads to a more widespread

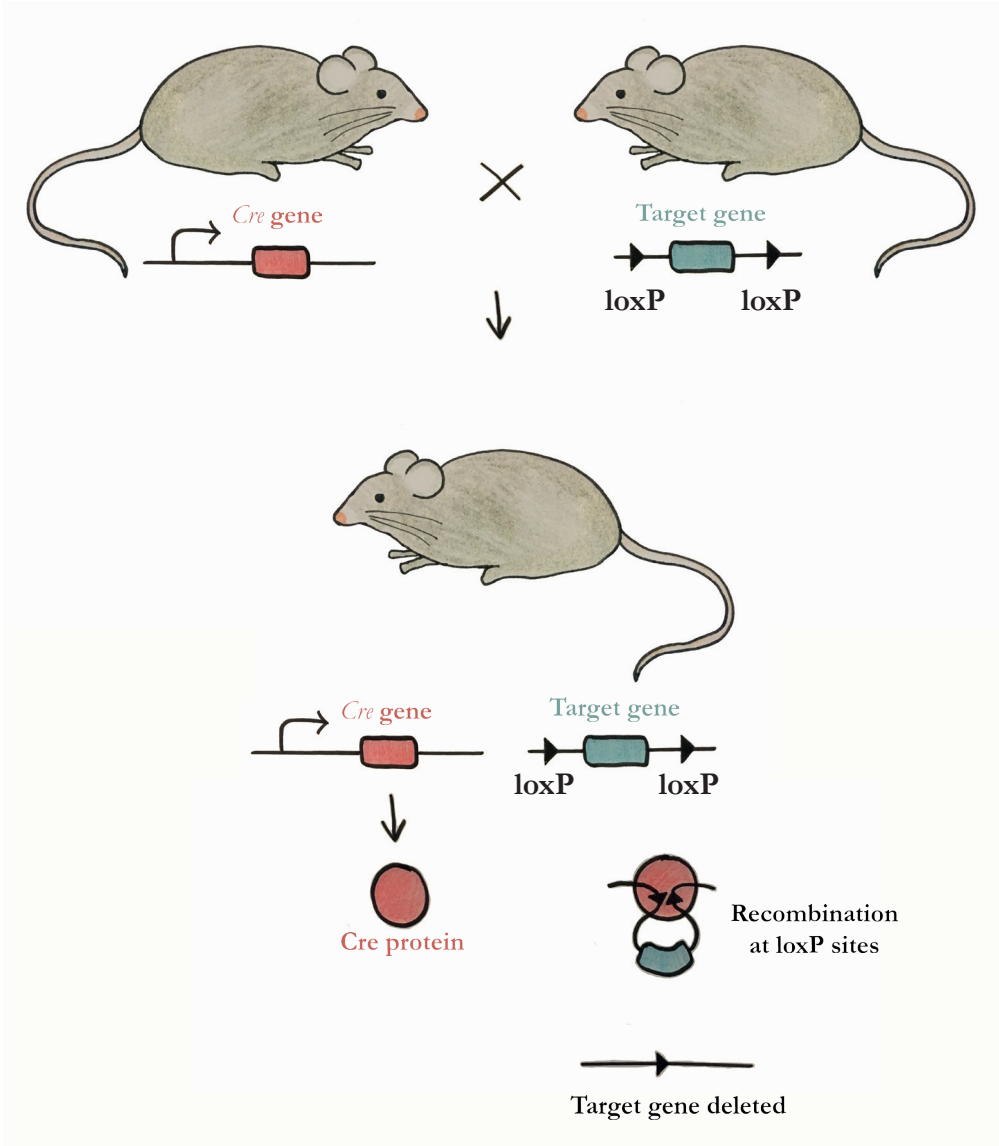

Fig 15. The Cre/loxP-system. A transgenic mouse with a tissue-specific Cre driver is bred with another transgenic mouse with a floxed target gene (upper two mice). In offspring (lower mouse) that has inherited both alleles, the Cre driver will perform recombination between the two loxP sites, thus deleting the target gene.

Cre-mediated recombination than desired (Heffner et al., 2012). This could be referred to as promoter leakiness. Another type of leaky or spontaneous activity is the activity of inducible Cre in absence of the inducing agent. In fact, this may also be advantageous as will be apparent discussing the results presented in Papers III and IV.

Even in cases of successful recombination in the desired tissue, the effect might not be the expected. There are findings of persistent protein expression following confirmed excision of a gene, due to episomal retention of the excised gene (Turlo, Gallaher, Vora, Laski, & Iruela-Arispe, 2010).

Nonetheless, the Cre/loxP-system is considered to be a robust system with high fidelity, but carefulness is still warranted when using this method.

6.2 LINEAGE TRACING AND REPORTERS

To study the dynamics of embryonic processes it is important to be able to trace cells in relation to some previous stages of development. In Paper I, the tracing of cells from different germ layers was an important matter. This was achieved by combining Cre drivers with reporter genes previously known to be germ layer-specific. *Sox17-2A-Cre* was used as a driver to express Cre in endoderm cells, in which the transcription factor Sox17 is ubiquitously expressed before organogenesis from the foregut (Engert et al., 2009). This was coupled to a reporter mouse strain that expressed β-galactosidase upon Cre-activation (Soriano, 1999). The design of the reporter gene is such that it contains a floxed stop codon, preventing its expression. Upon Cre recombination, β-galactosidase is expressed under the control of a ubiquitous promoter, ensuring stable expression as soon as the stop codon is removed. Tissues that are β-galactosidase positive, can be identified by their ability to use x-gal as a substrate to produce blue color, easily recognized in the light microscope. The advantage in this setup is that all progeny of a cell previously expressing Sox17, will express β-galactosidase. In our case, this provides a means to trace all cells that were once a part of the endoderm, regardless of their developmental fate.

Another reporter that was used in Papers I and IV is the double-fluorescent reporter *mTmG*, in which all cells express the red fluorescent protein Tomato before recombination. When Cre is active, it will excise the *Tomato* gene (*mT*) including a following stop-cassette, thus allowing for the expression of the green fluorescent protein GFP (mG) instead (Muzumdar, Tasic, Miyamichi, Li, & Luo, 2007). In both constructs, the encoded fluorescent proteins are membrane-bound (denoted by *m* in *mTmG*), providing sharp contours of the plasma membrane. This allows evaluation of cell shape changes in cells that co-express e.g. mutant Braf.

6.3 TRANSGENIC MOUSE MODELS OF THYROID CANCER

Mouse models are commonly used in the study of human cancers. One important approach is to use patient-derived xenograft (Pdx) models, in which tumor specimens from patients are implanted to immunodeficient mice, either subcutaneously or orthotopically (Einarsdottir et al., 2014). This allows for the study of human cancer cells *in vivo* and also to test targeted treatments with e.g. kinase inhibitors that may provide valuable information on the expected therapeutic response in patients. In a xenograft model of PTC, a human cancer cell line with Braf-mutant PTC showed tumorigensis in transplanted nude mice (D. Liu, Liu, Condouris, & Xing, 2007). One disadvantage with this approach is that the immune system of the host mouse needs to be suppressed, which makes it less faithful in mimicking the human cancer microenvironment.

Already in 1996, a mouse model of PTC was established, in which the RET/PTC fusion protein was expressed in follicular cells under control of the *Tg* promoter (Santoro et al., 1996). In this model, mice developed PTC slowly, but with a normal life-span.

Employing the Cre/loxP-system to generate genetically modified mice with activated oncogenes or inactivated tumor suppressor genes has generated novel information on mechanisms of endogenous tumor development and progression. One of the first mouse models of $Braf^{V600E}$-induced oncogenic transformation of cells was reported for thyroid (Knauf et al., 2005) and lung (Dankort et al., 2007), leading to PTC and lung cancer, respectively. In most instances, $Braf^{V600E}$-induced PTC used a strategy to constitutively express the floxed transgene from the onset of expression of the Cre-driving gene, mostly *Tg*, which implies that cells are transformed already in embryonic development (Charles, Iezza, Amendola, Dankort, & McMahon, 2011; Franco et al., 2011; Knauf et al., 2005; Orim et al., 2014). In Papers III and IV, a tamoxifen-inducible Cre driver, controlled by the thyroglobulin promoter (Undeutsch, Lof, Offermanns, & Kero, 2014), was combined with a floxed $Braf^{V600E}$ transgene to generate a more representative model of PTC development in adult mice.

”We ourselves feel that what we are doing is just a drop in the ocean. But the ocean would be less because of that missing drop”

Mother Teresa
(1910–1997)

7 Summary of results

SUMMARY OF RESULTS

7.1 PAPER I

Revising the embryonic origin of thyroid C cells in mice and humans

In paper I, the origin of mouse thyroid C cells was investigated. Experiments on chick-quail-chimeras in the 70s led to the conclusion that C cells in avian species are derived from neural crest cells (Polak et al., 1974). It was then believed that this was the case also in mammals. However, the conclusions from these experiments have been challenged by other studies indicating an endodermal origin of C cells in mice and warranting the closer study of their origin in mammals (Adams & Bronner-Fraser, 2009; Kameda et al., 2007).

Lineage tracing of cells derived from the neural crest was conducted, using the Cre/loxP-system in which Cre dependent activation of the double-fluorescent reporter mTmG was mediated by *Wnt1,* known to be expressed in early development only in the neural crest. Cells with a history of Wnt1 expression could thus be traced by their color conversion from red to green fluorescent protein (Tomato/GFP) and cells that had never expressed Wnt1 remained red. Neither follicular cells nor C cells were found in the GFP^+ cell population. This ruled out the possibility of a C cell origin from $Wnt1^+$ neural crest progeny. Next, the possibility of an origin in embryonic endoderm was tested through similar tracing of the fate of cells from $Sox17^+$ endoderm. This showed that C cells, identified by their expression of calcitonin, belonged to the progeny of $Sox17^+$ endoderm. The co-localization of Sox17-dependent expression of β-galactosidase and calcitonin was confirmed by laser confocal microscopy to ensure that the identifying signals were indeed obtained from the same cell. Thus, mouse thyroid C cells are endodermal.

Next, the expression of the endodermal markers Foxa1 and Foxa2 was investigated during development of the thyroid in mouse embryos and also in human MTC. Both were gradually induced in the developing ultimobran-

chial epithelium, whereas the thyroid primordium did not express Foxa1/2 at any developmental stage. This suggested the Foxa genes are functionally important for development of C cell precursors, correlating with their proliferation pattern. In tumor specimens, we found that Foxa1 had a growth-promoting role, and that tumor cells with invasive properties showed a coordinated loss of Foxa2 expression and E-cadherin that were re-expressed in metastases. This suggested that Foxa1 and Foxa2 may participate in MTC tumor progression and that MTC tumor cells undergo transient EMT.

7.2 PAPER II

Guidance of parafollicular cells (C cells) to the embryonic thyroid involves remodeling of basement membrane

It is presently unknown how thyroid C cells achieve their parafollicular position in the thyroid gland. Paper II gives a detailed description of the morphogenetic changes that the UB epithelium undergoes foregoing the entry of UB cell precursors into the embryonic thyroid. It also correlates functionally this process to basement membrane (BM) degradation.

Wild-type mouse embryos were studied from the budding of thyroid primordia in the pharyngeal endoderm to the onset of folliculogenesis in the primitive thyroid lobes (ages E9.5-15.5). In addition, *Pax8* null and *Nkx2-1* heterozygous mutants with altered UB development, to various degree independent of the midline thyroid, were investigated at E15.5. Both the midline thyroid (MT) and the UBs were found to be enveloped by a continuous BM during budding and early migration. However, prior to fusion, laminin staining and ultrastructural analysis showed that the BM of the UBs was gradually degraded. Localization of polarity markers (pericentrin and giantin) indicated loss of apical polarity and multilayering of the UB epithelium, albeit with preserved expression E-cadherin and thus the epithelial phenotype.

Laminin degradation on the UB surface occurred in a cell-autonomous way and required both Nkx2-1-alleles to be accomplished. This process facilitated UB-thyroid fusion and formation of a composite gland. Together, these findings provide a mechanism by which thyroid follicular cells and C cells meet and end up in such an intimate contact in the mature gland. Is also explains how embryonic C cells acquire a pro-migratory phenotype.

7.3 PAPER III

Follicular origin of tumor heterogeneity in a mouse model of sporadic papillary thyroid cancer

Existing mouse models of thyroid cancer commonly use genetic engineering to conditionally activate oncogenes or inactivate tumor suppressor genes of

known importance in humans (Kirschner, Qamri, Kari, & Ashtekar, 2016). This enables global expression in the entire gland, leading to rapid tumor development, which gives opportunities to study factors that modify tumor growth and progression. However, since most if not all thyrocytes are simultaneously transformed, present transgenic models lacking a natural microenvironment are far from mimicking the development of human thyroid cancer.

In paper III, we used an inducible mouse model of papillary thyroid cancer PTC, in which a *Braf*^{V600E} mutation was conditionally activated only in thyroid follicular cells expressing Cre under control of the *Tg* promoter. Induction with tamoxifen generated tumorous growth encompassing the entire gland. However, we discovered spontaneous Cre activation in the absence of tamoxifen and that microtumors developed multifocally, originating in single follicles, with closely located normal follicular tissue. Eventually, large tumors with signs of local invasion developed. Interestingly, tumor foci present in the same gland showed different phenotypes (classical, tall-cell, hobnail, cystic and solid variants) recognized in PTC in humans. Contrary to after tamoxifen-induced tumorigenesis, the sporadic tumors developed with normal thyroid hormone and TSH levels. Braf mutant tumor cells with hobnail appearance showed signs of oncogene-induced stress response (dilated endoplasmic reticulum and autophagosomes). Thus, this model enabled the detailed study of different stages in tumor development under conditions that closely resemble tumor development in humans.

7.4 PAPER IV

Tracing tumor-initiating cells in Braf^{V600E}-induced thyroid cancer

Although it has not been formally proven, the cell-of-origin in Braf^{V600E}-induced PTC is in all probability the differentiated thyroid follicular cell. However, the earliest events in tumor initiation are yet unknown. To further elaborate this issue, we generated a *Tg-Cre;Braf*$^{CA/+}$;Rosa26$^{mTmG/fl}$ mouse line in which mutant Braf and mTmG are conjointly expressed, thus labeling mutant cells and the progeny for direct identification in the fluorescence microscope.

First, spontaneous activation of Cre was studied in mice lacking *Braf*^{CA}. This showed that a few mG^{+} cells were present already before birth and that their numbers gradually increased until a majority of the follicular cells expressed GFP at 6 months of age. However, postnatal follicles (P10) mostly contained single mG^{+} cells without signs of clonal expansion, indicating that Cre activation was stochastic.

In *TgCre;Braf*$^{CA/+}$*;Rosa26*$^{mTmG/fl}$ mice, clonal expansion of mG^{+} cells was evident already at one month of age. Such mG^{+} clusters were increased in

number and size after 3-6 months and formed follicles in all instances. Eventually, they developed giant cyst-like structures limited by a monolayered epithelium that showed signs of inward papillary projections. To our knowledge, this is the first demonstration of pre-tumorous, histoarchitectural alterations induced by mutant Braf in the thyroid *in vivo*. Occurrence of single mG^+ cells with abberant morphology suggestive of OIS in high age mutant mice indicated differential susceptibility to oncogenic growth in the follicular cell population. The number of mG^+ cells markedly decreased in Braf mutant thyroids, suggesting spontaneous Cre activation was repressed and thus self-limiting to tracing.

Finally, the sporadic PTC model was tested in drug experiments, employing the tracing strategy to monitor early tumorigenesis. Animals with conditional Braf activation in the absence of tamoxifen were administered a diet with pellets containing the specific Braf inhibitor vemurafenib from weaning. Mutant littermates were put on a corresponding diet without vemurafenib. This showed that the thyroid size in treated animals was only 20 % of the much enlarged glands encountered in untreated mutants. Also, much of the morpohological changes induced by mutant Braf, were restored in treated animals. This experiment proves that $Braf^{V600E}$ is required and sufficient for thyroid tumor initiation.

”The more we are able to engage in enthusiastic disagreement with each other, the more we will be able to uncover the best in ourselves and each other”

Karen Kimsey-House
(b. 1954)

8 Discussion

Discussion

The first summarizing chapters have outlined the background for the studies that form the basis of this thesis. The four papers included in the thesis present the results from studies concerned with both embryonic development and tumor development. In this chapter, major findings will be briefly discussed in a broader context. For more detailed aspects, see individual papers.

8.1 FINDING CELL-OF-ORIGIN OF THYROID C CELLS AND MTC

Mechanisms that govern embryonic development and tumor initiation in the thyroid are far from fully understood. Knowing the origin of cells is important since it might favor the understanding of tumor development. Medullary thyroid cancer (MTC) is derived from C cells and belongs to the group of so-called neuroendocrine cancers. Since the origin of C cells has never before been definitively explored in mammals, paper I provides important insight by establishing the endoderm as the germ layer from which the C cells originate in mice. Since mice and humans share many developmental traits it is reasonable to assume that the same holds true for humans and probably the entire mammalian series.

The expression of forkhead box proteins Foxa1 and Foxa2 showed a pattern in human MTC that is in accordance with the properties they seem to confer in the embryonic thyroid regarding differentiation and growth. In view of numerous studies by others on the importance of these factors in both embryonic development and tumor development (Khoor, Stahlman, Johnson, Olson, & Whitsett, 2004; Minoo et al., 2007; Song, Washington, & Crawford, 2010), a differential expression of Foxa1 and Foxa2 as found in C cell precursors and MTC tumor cells probably reflects their roles in growth regulation and maintaining the epithelial phenotype. It is of particular interest that they may execute a similar function in invasion and metastatic spread.

Differentiation of thyroid C cells seemed to be spatiotemporally restricted during development with dedifferenti-

ation prevailing connected to events in which migratory properties are needed for the dissemination of C cells precursors into the thyroid proper. MTC is a tumor form that is prone to metastatic spread, which is probably an inherent trait. This possibly reflects the developmental course in which rearrangement of E-cadherin-based adhesion and persistent loss of apical polarity, as observed in paper II, are prominent features of the normal C cell phenotype. Notably, we observed that transient dedifferentiation, of MTC tumors, indicated by loss of Foxa2 and calcitonin expression took place in invasive tumor cells that underwent EMT. Speculatively, the inborn ability to degrade basement membranes, as found in embryonic C cells emerging from the UBs, might be reactivated in MTC facilitating tumor cell invasiveness. The expression of calcitonin among normal cells is unique for thyroid C cells, and measurement of calcitonin blood levels is valuable as a biomarker in MTC (Bae, Schaab, & Kratzsch, 2015). However, calcitonin expression is only present in differentiated cells and the progression of a tumor to a less differentiated state, associated with higher level of aggressiveness is a plausible subject for continued studies.

Lineage tracing has provided powerful methods to investigate and determine the fate of different cell populations during development (Kretzschmar & Watt, 2012). The origin of C cells in endoderm explains several notions that were previously hard to understand. One of them is that C cells have been found in some patient with DiGeorge syndrome, in which the fourth pharyngeal pouches and consequently, the UBs do not form (Pueblitz, Weinberg, & Albores-Saavedra, 1993). Also intringuingly, MTC has been found in the lingual thyroid of a patient, in which no orthotopic thyroid was found (Yady, Singh, Singh, & Aggarwal, 2008). A shared endodermal origin of the two cell lineages makes it more plausible that early progenitors might be potent of developing into both cell types.

Another important matter, on which the endodermal origin of C cells could shed light, is the fact that mutations in the *RET* proto-oncogene are found in both PTC and MTC, while being rare in other tumor forms (Romei et al., 2016). Also intriguingly, is the observation that there are cases of mixed medullary-follicular thyroid carcinoma (MMFTC) present at higher rate than could be expected by random co-occurrence of two independent cancers (Matias-Guiu, 1999; Ueki et al., 2011). A shared endodermal origin is compatible with a susceptibility of both cell types to transformation by the same oncogenic germ line mutation. Indeed, there are hereditary forms of MTC, in which mixed tumors develop already in childhood, possibly from a stem cell, capable of differentiation into both follicular and C cells (Goyal, Nada, Rao Katragadda, & Radotra Bishan, 2006; Kovacs Christopher, Masé Robert, Kovacs, Nguyen, & Chik Constance, 2006; M. Nilsson & Williams, 2016).

There are some lessons to learn from the concept of the neuroendocrine system, which emerged to describe endocrine tissues that were thought to have a shared embryonic origin in the neural crest. First, the results of a study are always limited by the method employed, and the development of new techniques is constantly giving us previously unforeseen opportunities to study biological phenomena. The avian chimera model was seminal to investigate the fate of NCCs and rendered very important knowledge about embryonic development, although some of the conclusions from these studies now have proven faulty or at least not valid in mammals. Second, there is important cross-talk between different tissues during development and without doubt, the NC provides important contributions, not only to the transient structure of the pharyngeal apparatus, but also in the signaling network that is important also for regulating the development of surrounding tissues. Understanding the migratory properties of the NCCs could also aid in the search for mechanisms that govern the grade of differentiation and proneness to invasiveness in transformed cells. Third, a concept that originally evolved based on an assumed shared embryonic origin, might still be relevant in describing shared phenotypic characteristics of tissues and tumors derived from cells with distinct gene expression signatures.

8.2 TUMOR INITIATION IN PTC

Cells of the same lineage in a given tissue are not all homogeneous. They display a heterogeneity regarding the level of differentiation and growth-prone abilities that influence the cell phenotype and possibly the response to various stimuli, although the microenvironment in which they reside is similar. However, to what extent if any such heterogeneities may explain why the same oncogenic event and constitutive activation of identical intracellular signaling pathway confer development of different tumor phenotypes is unknown. In Papers III and IV, we characterized the sporadic activation of Cre in a mouse model of $Braf^{V600E}$-induced PTC. In most models that use the Cre/loxP-system to achieve spatiotemporal control of a genetic event, sporadic recombination in absence of the inducing agent is considered to be a disadvantage. In our studies, we took advantage of this ugly duckling. We discovered that this proved a unique possibility to study tumor development in an environment that is a closer mimic of human PTC development than in previous models, in which global activation of an oncogenic mutation, no matter if it is constitutively or induced, transforms the entire thyroid gland with general and profound effects on epithelial morphology and hormone status. Our mice were euthyroid, ruling out the effect of elevated TSH signaling as a growth-stimulating factor. We found that a plethora of PTC histotypes de-

veloped multifocally in the same gland over a considerable time period of the mouse lifetime that may correspond to the natural course of tumor development in humans. Although additional genetic aberrations may occur explaining differences in clonal growth characteristics, these studies provide the first insight in the evolution of thyroid cancer from tumor initiation in the follicular context to the development of overt, macroscopic tumors.

8.3 THE INSTRUMENTAL SPONTANEOUS ACTIVATION OF CRE

The spontaneous activation of Cre in the absence of induction by tamoxifen was investigated with the double-fluorescent reporter mTmG in Paper IV, and we were thus able to quantify the pace at which sporadic tumor initiation and development occurred. In the normal thyroid, we found that Cre activation started already at the embryonic stage E18.5, only three days after onset of Tg expression. Gradually, more cells converted to the pool of mG^+ thyroid follicular cells. They mostly appeared as single green fluorescent cells although, rarely, mG^+ cells neighboring each other were encountered. Since the thyroid is substantially enlarged postnatally due to follicular cell proliferation and new follicles are likely to be added to those already formed in embryonic development, the limited number of mG^+ cell pairs appearing before the age of one month suggested that recruitment by cell division within the mG^+ pool contributed little and that Cre was mostly activated stochastically. Importantly, this showed that sporadic Cre activity is a phenomenon that eventually encompasses a majority of cells in the normal gland, but that individual mG^+ cell are most often encountered with only mT^+ neighbors. This allows the tracing of individual mG^+ progenies and their clonal expansion upon a targeted growth stimulus.

By combining *TgCre;Rosa26*$^{mTmG/fl}$ mice with *Braf*^{CA} mice, we were able to trace, for the first time, the destiny of oncogene-.activated, differentiated thyroid follicular cells *in vivo* in the natural microenvironment during postnatal thyroid growth and in the adult gland in which cell renewal normally is very slow (Coclet, Foureau, Ketelbant, Galand, & Dumont J, 1989). Assuming the concept of clonal growth from a single mutant cell, our studies clearly showed that Braf mutant thyroid cells are able to form follicles by planar growth, thus indicating that both epithelial and follicular integrity were preserved. This further showed that planar growth eventually leading to a highly convoluted follicular epithelium formed the basis of papillary formations featuring the classical PTC variant.

We expected to find tumor growth only among mG^+ cells. However, it was evident that also the mT^+ epithelium exhibited changes resembling of tumor development. The definite answer to why this happened is still elusive and possible explanations are discussed in Paper IV. Without induced activation

of a Cre driver and with a combination of several floxed alleles, it is apparently crucially important to take into consideration the possibility of a stochastic pattern of recombination events that not necessarily activates all transgenes simultaneously in the same cell. Also, down-regulation of Tg and thus Cre expression by constitutive activation of the MAPK pathway opens for a self-limiting process that may hinder activation of mTmG in Braf mutant cells. The fact that mT^+ neoplasias dominated over mG^+ tumors are in line with this possibility.

Several existing mouse models use combinations of floxed genes to study the effects of multiple genetic events and their impact on tumor development. For example, inactivation of the tumor suppressor gene *TP53* has been shown to be important in the development of anaplastic thyroid cancer (ATC). In a mouse model similar to ours, activation of $Braf^{V600E}$ in combination with inactivated p53 led to progression from PTC to ATC (McFadden et al., 2014). Using a similar model without induction would be an attractive way to study also ATC in a more realistic way. However, the results from Paper IV shows that such a strategy needs to take into consideration the fact that the desired multiple recombination events might not take place within the same cell.

Regardless technical shortcomings, tumor lineage tracing in the sporadic model still provides important opportunities to study early events during tumor development and the interactions between tumor-initiated cells and their microenvironment. Also, mutant cells that show signs of OIS are readily detectable by mTmG reporter activation, making it possible to identify and study their fate more closely.

”A hundred suspicions don’t make a proof”

Fyodor Dostoyevsky, in Crime and Punishment
(1821–1881)

9 Conclusions

Conclusions

The thesis work may be concluded around two themes based on cell-of-origin of thyroid cancer and their normal endocrine counterparts as follows:

C cells – MTC

- Thyroid C cells originate in pharyngeal endoderm, rejecting the previous concept of a neural crest origin
- Degradation of laminin promotes C cell entry to the embryonic thyroid manufactured by a Nkx2-1 dependent mechanism in the ultimobranchial epithelium
- The pro-migratory properties of epithelial C cells are established during organogenesis by loss of apical polarity
- Foxa1 and Foxa2 likely exert different roles in promoting growth and differentiation of embryonic C cells
- Human medullary thyroid carcinoma cells differentially express Foxa1 and Fox2 in a way that correlates with tumor growth and invasion

Follicular cells – PTC

- Spontaneous activation of iTgCre recombinase in an inducible mouse model targeting $Braf^{V600E}$ to the thyroid, generates multifocal tumors in euthyroid mice
- This model confers sporadic development of papillary thyroid cancer subtypes in the normal thyroid microenvironment
- Tracing tumor initiation by the double-fluorescent reporter (mTmG) enables the study of early events in $Braf^{V600E}$-induced thyroid cancer
- The sporadic thyroid cancer model is suitable for studies of targeted drug therapy

”There is no better boost in the present than an invitation into the future”

Caroline Kepnes
(b 1976)

10 Future perspectives

Future perspectives

My work is by no means complete or finished. There are a lot of possible ways to continue from the achievements so far.

Regarding the tumor project involving mTmG it is vitally important to investigate the correlation between Braf activation and color conversion. This involves understanding the kinetic mechanisms that affect Cre activation and the possibility of a double recombination in the same cell. Without understanding the extent to which Cre both enacts oncogenic activation and color conversion it its impossible to appreciate the direct effects of the oncogene activation compared to the effects that tumor-initiated cells have on their environment.

Regardless of this, our non-induced mouse model of PTC can be used to address a number of interesting questions. It provides a platform for a diversity of morphological and molecular investigations.

Specimens from animals in the *Tg-Cre;Braf*^{CA}*;Rosa26*$^{mTmG/fl}$-model of PTC could be immunostained with a blue fluorochrome. This allows for a number of questions being addressed to a model in which monoclonal expansion of Braf mutant cells is available for investigation. For example immunostaining for laminin would be a way to pinpoint the changes in BM distribution that are involved in invasiveness.

The possibility to take advantage of sporadic Cre activity could very well be used in other mouse models with leakiness in the Cre driver. Careful characterization of the sporadic activity should be undertaken and if combining several floxed transgenes, the correlation between recombination events at the different alleles should be determined.

In the end, the most important outcome is improved treatment for patients with thyroid cancer. Hopefully our model can help both in finding ideas for new treatments and for evaluation of the efficacy of drug candidates.

Ongoing work in our lab includes combining the PTC model with conditional inactivation of p53, which is expected to confer progression to ATC. As mentioned in the Discussion section,

this requires careful assessment of the correlation between recombination events. Since we use the mTmG reporter, tumor tissue could be subjected to fluorescent-activated cell sorting to isolate different cell populations and analyze genetic profiles and expression patterns.

Others that work with the Cre/loxP-system could probably benefit from our approach to take advantage of the spontaneous Cre leakiness in non-induced mice to elaborate tumor development in a more natural setting than provided by global oncogene activation.

There is still a lot of work to be done!

”We meet no ordinary people in our lives”

C.S. Lewis
(1898–1963)

11 Acknowledgements

Acknowledgements

Any long journey is lonesome without company. My journey as a PhD student would not have been possible to complete without the support and the company of many other people. Also, it would not have been nearly as fruitful and nice. In this section, I want to express my gratitude to all of you who endured joining me for parts or the whole of this journey. Some of you were deeply involved yourselves in my projects, some of you helped making other parts of my life meaningful, and some of you did both.
I would not be were I am today had it not been for you!

Mikael Nilsson: My main supervisor with a true passion for thyroid research, good food and Puccini. Thank you for accepting me as a PhD student in your lab. This has been a long, sometimes hard, and indeed fruitful time and I am grateful to you for letting me develop as a PhD student and scientist, and for helping me to develop independence and find my own ways.

Bengt R Johansson: My co-supervisor who introduced me to the fascinating world of research, electron microscopy, and anatomy teaching. Thank you for generous support and mentorship in so many ways.

Henrik Fagman: My second co-supervisor who is always ready to answer an e-mail at any time, day or night. Thanks for guidance, support and help to see the broader perspectives.

Ulf Bagge: Thanks for being my mentor, asking questions, reading manuscripts, giving guidance and for nice times at the coffee table solving cross-words.

Camilla Ingeson-Carlsson: Former member of the group who was important as a support for me when I came to the group. Your enthusiasm and energy are so inspiring and you really have a talent for keeping spirits up in times of struggle. A special thank for accepting my wish to have you as toastmaster of my dissertation party.

Shawn Liang: Much appreciated co-worker and friend. We came to the lab the same year and we finish almost simultaneously. Always steady, calm and extremely idle.

Elin Schoultz: Former class-mate, friend since many years and partner in science. Thanks for long-standing friendship, support in hard times and good co-operation. My (unfinished) projects are your projects!

Carmen Moccia: Coming as a master student from Naples at the time when I needed you the most. Thanks for bringing new energy and inspiration, for teaching me Italian, and for sharing time, thoughts and great Italian food.

Therese Carlsson: Excellent lab technician who never flatters anyone, but who has a truly warm and kind heart. Thank you for good technichal support, for sharing your wonderful personality and for showing how to deal with so many things in a good way.

Yvonne Josefsson: My laboratory "mother" who took good care of me when I first came to the electron microscopy unit. Efficient, accurate, and always helpful.

Kanita Cukur: Never agrees just to be polite, not afraid of telling a harsh truth, and sincerely faithful to her friends. Whenever you need a sunny day, she is ready to take a walk with her raincoat. I have a hypothesis that if everybody had a person like Kanita in their lives, the effect on public health would be significantly fewer depressions.

Johanna Höög and **Per Widlund**: Thanks for showing me deeper parts of the scientific ocean and helping me to swim in it. You are truly inspiring!

Magnus Braide: Professor of anatomy and teaching collegue. Thanks for giving me the opportunity to teach and for allowing me to try new ideas and projects in teaching. While project leaders change, your patience and good sense of humor never end.

Jonna Sjöholm: Thanks for taking good care of all our animals and for generously sharing your knowledge and techincal skills regarding laboratory animals.

Anna Linder: My dear friend who is the incarnation of hard work, never giving up despite hardships and uphills.

Karin Larsson: My office-mate who is the sunshine of floor 4. Always with a smile and always ready to help or discuss any matter. Your long experience has been so valuable to me.

Maryam Kakay Afshari: Always supportive, always amiable, always ready to offer fruit, nuts and Persian food.

Anna Wenger: You saved many evenings when I was at risk of getting stuck at the lab too late. Thanks for walking company and nice discussions.

Malin Hagberg Thulin: One of the first persons I met at SCC, always kind and supportive. It always feels safe to talk to someone who has gone through the same struggles.

Anne Uv: Study of director at the institute, often attending the same meetings as I. You are always determined to solve things in the best way. Thanks for all support!

I would also like to thank:

All other former and present friends at Sahlgrenska Cancer Center:

Agota Tuszi, Anna Danielsson, Anna Nordin, Berglind Ósk Einarsdóttir, Gustav Johansson, Junchi Huang, Karin Welén, Mamen Leiva Arrabal, Melita Kaltak, Soheila Doletabadi, Somsundar Veppil Muralidharan, Susann Busch

Sebastian Malmström: We started with a choral friendship, which developed to also include science, vocation, cats and other important things in life. A special thank you for taking my picture for the back cover. You made me look better than reality!

Sankt Pauli congregation for being my spiritual home and all friends there for being an extra family.

Medical students for asking me questions that keep me alert and wanting for more knowledge.

Sahlgrenska Akademins Studentkår and **Doktorandrådet** for giving me opportunities to learn so much more about organizations and the prerequisities for good working conditions.

Friends from different times and places. You are too many to mention each of you but I feel so blessed to have you all in my life.

My dear family: My parents **Eva** and **Erland** for all your love and care; my dear brothers **Edvard**, **Erik**, **Emil** and **Elis**, who have been my friends and role-models, always ready to support me; my sisters-in-law and my wonderful nephews and nieces, who remind me of the really important things in life. Aunts, uncles cousins and your families for being precious long-term parts of my life.

Daniel, my dearly beloved! Thanks for your support, encouragement, kindness and love. You make all the difference every day!

I also want to express my sincere gratitude to **Göteborgs Läkaresällskap** and **Assar Gabrielssons fond** for providing financial support, allowing me to achieve so much more in science.

SDG

”If I have seen further it is by standing on the shoulders of Giants”

Isaac Newton
(1642–1727)

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