ReCREating BRAF-driven thyroid and lung cancer in mice

Elin Schoultz

Department of Medical Chemistry and Cell Biology
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2022

Cover illustration: “A tree of life and death”

A micrograph of an intratracheal papillary tumor - with a fancy filter

Design by Elin Schoultz and Junchi Huang

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elin.schoultz@gu.se

ISBN 978-91-8009-805-2 (PRINT)
ISBN 978-91-8009-806-9 (PDF)
http://hdl.handle.net/2077/70942

Printed in Gothenburg, Sweden 2022
Printed by Stema

I’d rather be a comma, than a full stop

-*Christopher Martin*

To Grandpa Allan

ABSTRACT

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer with a 3:1 female/male incidence. PTC is caused by oncogenic *BRAF* mutation encoding $BRAF^{V600E}$ in 50% of cases. Prognosis is mostly excellent post-surgery and radioiodine therapy, but 15% of PTC patients with clinical tumors die of the disease. Transgenic mouse models are invaluable tools in dissecting the mechanisms of thyroid tumor progression, and to monitor novel targeted drug treatments *in vivo*. Conditional expression of $BRAF^{V600E}$ using a thyroid specific Cre driver e.g., the thyroglobulin (*Tg*) promoter, can be used to activate mutant Braf ($Braf^{CA}$) specifically in the thyroid and with temporal control using tamoxifen inducible Cre. However, an inborn problem with this procedure is that nearly all thyroid cells are synchronously oncogene-activated. This causes hypothyroidism and unphysiologically high levels of circulating TSH that is goitrogenic, making it difficult to investigate tumor clonality confounded by reactive hyperplasia. In paper I, we developed a new PTC model based on stochastic BRAF activation (due to spontaneous Cre activity in the absence of tamoxifen) by which tumors developed in a normal microenvironment and with maintained systemic thyroid function. Originating from a single follicle, individual tumors had different histologic phenotypes and were initially oligoclonal identified by lineage tracing. We applied this model in paper III to evaluate drug responses to a BRAF-inhibitor (a vemurafenib analog) and found that female mutant mice recovered poorer in thyroid gene expression (*Slc5a5* and *Tshr*) than males and developed larger tumors that progressed more with long-term drug treatment. Analysis of cytokine expression in paper IV revealed differential cytokine expression indicating tumor heterogeneity distinguished by level of inflammation, and that the tumor cells themselves secreted cytokines (IL-1β, IL-6 and TNF-α) in early tumor development. Finally, we confirmed that targeted oncogene activation without induction can generate sporadic tumorigenesis in other tissues. In paper II, using *Nkx2.1*, a transcription factor shared by thyroid and lung, as Cre driver, mutant BRAF independently caused both thyroid and non-small cell lung carcinomas with different growth and progression features consistent with modulation of oncogene activity in an organ-specific fashion. This represents the first mouse model in which lung adenomas progress to adenocarcinomas due to BRAF mutation.

Keywords: thyroid cancer, BRAF mutation, transgenic models, clonal tracing, BRAF inhibitor, sex differences, lung cancer

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SAMMANFATTNING PÅ SVENSKA

Sköldkörtelns vanligaste cancerform är papillär thyroideacancer (PTC) där >50% av fallen har en s k *BRAF*-mutation. Denna mutation orsakar en onormalt hög aktivering i ett cellulärt signalsystem, vilket leder till ohämmad celldelning och tillväxt, på liknande sätt som vid malignt melanom (hudcancer) och colon- (tjocktarms-) cancer. Mutationen förekommer även vid tumörutveckling i lunga, dock i mycket mindre grad (<5%), och är associerad med utveckling av aggressivt växande adenocarcinom. Prognosen vid PTC är i allmänhet god med kirurgi och radiojodbehandling, eftersom thyroideaceller är specialiserade på att ta upp jod. Trots detta drabbas vissa patienter av återfall med terapiresistent sjukdom och sämre prognos. Vid lungcancer med *BRAF*-mutation är prognosen sämre, framför allt då kirurgi oftast inte är möjlig samt att återfallsrisk och resistensutveckling är hög.

Med hjälp av en genetiskt modifierad musmodell för *BRAF*-muterad PTC studerar vi tumörutveckling och hur olika tumöregenskaper påverkar progression mot avancerad sjukdom i delarbete I, III och IV. Vi studerar även tumörutveckling i lunga med samma Braf-mutation i delarbete II. Både lunga och thyroidea kommer från samma embryonala anlag, och har flera utvecklingsmekanismer gemensamt, även om organens slutliga utseende och funktion skiljer sig.

Det finns flera olika musmodeller för såväl PTC som adenocarcinom i lunga. Problemet med många av dessa är att de bygger på samtidig aktivering av en mutation i väldigt många celler. Tumörutveckling och -tillväxt sker snabbt och aggressivt, varefter djuren vid PTC blir hypothyroida (hormontillverkningen i sköldkörteln upphör) eller vid lungcancer drabbas av andningssvikt och dör snabbt. Våra musmodeller bygger på att ett mindre antal celler kommer aktivera *BRAF*-mutation och orsaka fokala förändringar med bibehållen mikromiljö. Detta efterliknar cancerutveckling hos människa bättre, och gör det möjligt att studera tumörskillnader både mellan olika kön, individer, organ samt inom ett och samma organ. Djuren blir inte hypothyroida, lever längre och mer avancerade stadier kan analyseras.

En väldigt viktig fråga är hur stor betydelse tidpunkten för när en cell blir muterad har för dess förmåga till tumörutveckling? I delarbete I och II introduceras *BRAF*-mutationen vid olika tidpunkter under sköldkörtelns utveckling och vi undersöker tumörutvecklingen vid olika åldrar, både embryonalt och hos unga/vuxna djur, för att försöka besvara detta. I delarbete II studerar vi om tumörutveckling i thyroidea och lunga skiljer sig när

mutationen är densamma. Vidare undersöker vi för PTC eventuella skillnader mellan hon- och handjur i delarbete I och III då PTC och *BRAF*-muterad lungcancer hos människa är vanligare bland kvinnor.

I delarbete I, III och IV utvärderar vi behandlingseffekt av den specifika BRAF-hämmaren vemurafenib i PTC-modellen avseende tumörvolym, ifall behandling kan göra thyroideacellerna mer mottagliga för radiojodbehandling samt om det finns könsskillnader. Våra resultat i delarbete I och III visar behandlingseffekt på kort sikt, men med längre behandlingstid är resistensutvecklingen tydlig. Vi ser tydliga könsskillnader i behandlingsresultat i delarbete III, där behandlingseffekten är sämre hos hondjur på både kort och lång sikt.

Eftersom mikromiljön är bevarad i dessa musmodeller får vi möjlighet att studera reaktionen i vävnaden runtomkring tumörerna, vilken visat sig ha stor betydelse när det gäller tumörprogression och utveckling av behandlingsresistens hos människa. Signalering mellan tumörceller, bindvävsceller och immunceller kan till exempel påverka mikromiljön och underlätta invasion och metastasering av tumörceller. I delarbete IV undersöker vi några inflammatoriska komponenter i tumörer vid olika åldrar, eventuella skillnader i uttryck inom en och samma tumör, samt före/efter behandling med vemurafenib.

LIST OF PAPERS

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

I. **Elin Schoultz**, Ellen Johansson, Carmen Moccia, Iva Jakubikova, Naveen Ravi, Shawn Liang, Therese Carlsson, Mikael Montelius, Konrad Patyra, Jukka Kero, Kajsa Paulsson, Henrik Fagman, Martin O. Bergö, Mikael Nilsson

Tissue architecture delineates field cancerization in *BRAF*^{V600E}-induced tumor development

Disease Models & Mechanisms. 2022 Feb 1; 15(2)

II. **Elin Schoultz**, Shawn Liang, Therese Carlsson, Stefan Filges, Anders Ståhlberg, Henrik Fagman, Clotilde Wiel, Volkan Sayin, Mikael Nilsson

Stochastic oncogene targeting of *Nkx2.1*-lineage cells differentially recapitulates BRAF-driven tumor development and progression in lung and thyroid

Manuscript

III. **Elin Schoultz**, Carmen Moccia, Thomas Ramo, Therese Carlsson, Mikael Montelius, Henrik Fagman, Martin O. Bergö, Mikael Nilsson

Sex bias of BRAF-inhibitor therapy in mice with papillary thyroid cancer

Manuscript

IV. **Elin Schoultz**, Thomas Ramo, Carmen Moccia, Mikael Nilsson

Heterogeneity of a *BRAF*^{V600E}-induced cancer inflammation in a mouse model of sporadic thyroid tumorigenesis

Manuscript

CONTENTS

ABBREVIATIONS

AMP	Adenosine monophosphate
ATA	American thyroid association
ATC	Anaplastic thyroid carcinoma
Cre	Causes recombination
CC10	Club cell secretory protein 10
CSC	Cancer stem cell
Cxcr4	Chemokine receptor type 4
DUOX	NADPH oxidase
EGFR	Epidermal growth factor
ER	Estrogen receptor
GPER1/GPR30	G-protein coupled estrogen receptor
FoxA1	Forkhead homeobox gene A1
FoxA2	Forkhead homeobox gene A2
FNAC	Fine needle aspiration cytology
FTC	Follicular thyroid cancer
IHC	Immunohistochemistry
Il1b	Interleukin-1 beta
Il6	Interleukin-6
LBD	Ligand binding domain
loxP	Locus of x-over of bacteriophage P1
MAPK	Mitogen-activated protein kinase

MCT8	Mono carboxyl transporter 8
Mmp2	Matrix metalloproteinase 2
MTC	Medullary thyroid cancer
mTmG	Membrane-bound Tomato/membrane-bound GFP
NIS	Sodium-iodide symporter, encoded by *SLC5A5*
Nkx2.1	NK homeobox gene 2.1
Pax8	Paired homeobox gene 8
PDTC	Poorly differentiated thyroid cancer
PLX4720	Analog of vemurafenib; BRAF inhibitor
PTC	Papillary thyroid cancer
RAF	Rapidly growing fibrosarcoma
RAI	Radioactive iodine
RAS	Rat sarcoma virus
RTK	Receptor tyrosine kinase
SiMSen-Seq	Simple multiplexed sensitive mutation detection using sequencing
TCGA	The cancer genome atlas
TERT	Telomerase reverse transcriptase
TIC	Tumor initiating cell
TKI	Tyrosine kinase inhibitor
T3	Triiodothyronine
T4	Thyroxine/tetraiodothyronine
TG	Thyroglobulin
TGIF	Thank God It's Friday

Thbs1	Thrombospondin-1
TNM	Tumor Node Metastasis
TPO	Thyroperoxidase
TSH	Thyroid stimulating hormone
TSHR	TSH receptor
TTF-1	Thyroid transcription factor 1
Tnfa	Tumor necrosis factor alpha
UB	Ultimobranchial body
WHO	World health organization

PREFACE

When I started the medical program, some 100 years ago it seems, my target image was to become an orthopaedic surgeon, and I was sure about two things: I was not going to do any research and I was not going to teach (like my parents). Fortunately, one can change! Now, I'm finishing my PhD program in basic medical science and I'm teaching anatomy and histology since many years…

This thesis is about specific cancer development in two of our organs: the thyroid, a hormone producing gland regulating metabolism in every human cell, and the lungs. The cancer investigated in this thesis is caused by a mutation in the *BRAF* gene, crucial to molecular signaling regulating cellular growth, cell differentiation state and survival. To recapitulate the sporadic tumor development, we use a genetically engineered mouse model in a way that, to our knowledge, mimics the human situation far better than previous models.

All the cells building up our bodies follow strictly regulated programs enabling them to fulfill their ultimate purposes, maintaining homeostasis. Although a mutation might be a seemingly small error within the genome - like the mere switch of one amino acid to another - the consequences for the subjected cell and organism might be grave, depending on when, where and in whom the mutation was initiated.

1 INTRODUCTION

1.1 Common grounds, segregated end products

In the 3rd human gestational week, shaping of the final organism is commenced by growth factor gradient defined body axes: cranio-caudal, medial-lateral, left-right, and later antero-posterior. Three primitive tissue/cell or primary germ (embryonic cell) layers – endoderm, ectoderm, and mesoderm – of the developing embryo are formed during a process called *gastrulation* [1, 2]. During the subsequent phases between 4th-8th weeks, the germ layers will cooperate by patterning the different layers, perform regional specification and eventually give rise to separate tissues and organs at their respective locations during *morphogenesis/organogenesis* [3, 4]. The chronological mouse equivalent for gastrulation and organogenesis is embryonic day (E)6.5-8.5 and 8.5-15, respectively.

From the endodermal germ layer the thyroid gland, respiratory system, parathyroid gland, thymus, gastrointestinal system, bladder, and urethra will be formed. The mesoderm is the origin of the heart and circulatory system, blood, bone, cartilage, muscles, lymphatic system, kidney, adrenal cortex, spleen, and ureters. The nervous system, parts of the sensory organs and the skin with its appendages is derived from the ectoderm.

The gastrulation and morphogenetic processes are accomplished through a series of dynamic cellular processes involving changes in size, shape, number, position, intercellular connections, and migration. After the 8th week, the embryo now called a fetus, and during the remaining time in utero until birth (approximately 40 weeks in humans and 20 days in mice) functional maturation and growth of the formed organs will take place.

It has been shown that the developmental processes for multiple organs are phylogenetically conserved and very similar between several species, which is why we can use animal models for detailed studies of normal organ development as well as pathological conditions [5, 6]. The developmental stages described in the text are shared between mice and men although the timings differ [7].

During the 4th gestational week, the trilaminar embryonic disc will expand and fold. The endoderm will assume the shape of a tubelike structure, the gut tube,

which is subdivided into a cranial part: the foregut endoderm, a middle part: the midgut endoderm, and a caudal part: the hindgut endoderm [8]. In the cranial part the mouth orifice will form and in the most caudal the anal and urogenital openings develop.

In the anterior part of the foregut endoderm, the pharyngeal apparatus - pharyngeal arches (with cranial nerves and pharyngeal aortic arch derived arteries), clefts and pouches - will develop sequentially during the 4th and 5th week [9] with contributions from all three primary germ layers. The pharyngeal endoderm is the primary contributor to the parenchyme of the future thyroid and lung.

In this thesis we investigate the initiation, development, and progression of tumors prevalent in humans, by in vivo studies of mouse thyroid and lung using genetically engineered mice. The thyroid and the lung start to develop almost simultaneously, in very close proximity and both are of endodermal origin. However, their respective “final products” are very different. It is therefore a proper initiation to provide basic knowledge of the anatomy, morphology function and development of the organ systems, to understand (some of) the mechanisms in cancer development. The thyroid will be described first regarding these aspects, and thereafter the lung.

1.2 THE THYROID GLAND

1.2.1 Anatomy and function

Even though the thyroid gland was named after its resemblance of a Greek shield, it is more often referred to as butterfly shaped. The first recognized anatomical description and illustration of a thyroid gland was made by Andreas Vesalius 1543 in *De corporis humani fabrica libri septem*, although Leonardo da Vinci already had depicted its contour in the painting *The Madonna of the Carnation* already in 1500 (I can't help but wonder if she has a goiter). However, it was Thomas Wharton, English medical doctor and anatomist, that eventually named and fully described the anatomy of the **glandula thyreoidea** in *De corporis humani fabrica libri septem* 1656.

In mice and humans, the thyroid is a bilobed gland located in the anterior neck, below the thyroid cartilage/larynx, with an isthmus connecting the lobes at the level of the 3rd tracheal (cartilaginous) ring (*Fig.1*). It is an endocrine organ, producing the hormones thyroxin (T4), the main product and circulating hormone, and triiodothyronine (T3), the biologically active substance enabled by peripheral conversion of T4.

All cells throughout the whole human body are targets of thyroid hormones, that mainly regulate metabolism and somatic growth, especially important for development of the nervous system [10]. The levels of free hormones are regulated by the Hypothalamus-Pituitary-Thyroid (HPT)-axis, a negative feedback system orchestrated by stimulatory signals from the pituitary to the thyroid as Thyroid Stimulating Hormone (TSH) [11]. Disturbance of the hormonal equilibrium ultimately results in either hypo- or hyperthyroidism, i.e. decreased or increased metabolism causing various symptoms [12].

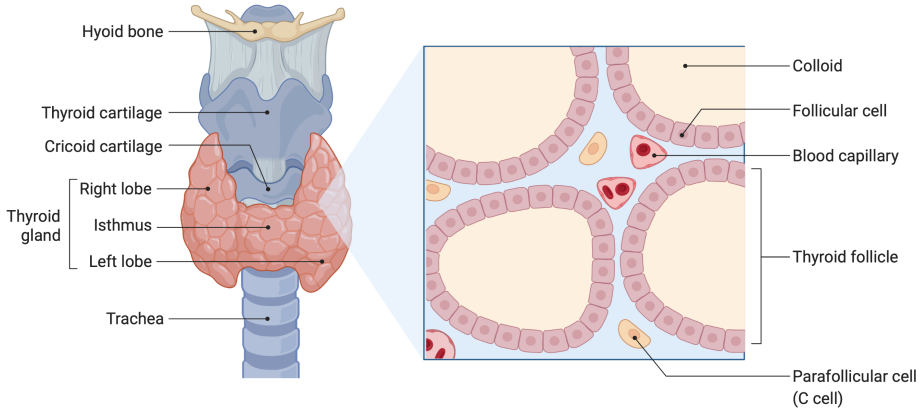

Fig.1. Thyroid gland anatomy and histology

The functional units of the thyroid gland are numerous ovoid- or spherically shaped follicles (*Fig.1*). They are formed by a single layer of cuboid epithelium called follicular cells, resting on a basement membrane, surrounding a fluid-filled lumen. The follicular cells (or thyrocytes) are polarized, with a basal aspect facing the surrounding tissue interstitium containing a highly vascularized stroma, while the apical surface is directed towards the follicular lumen [13]. The apical and basal compartments are kept separated by intercellular tight junctions, while adherence junctions with the epithelial cell adhesion molecule E-cadherin contributes to the adhesion of adjacent cells [14]. The follicular lumen contains the colloid, a proteinaceous fluid with mainly concentrated Thyroglobulin (TG), produced by the follicular cells, and a precursor reservoir of T3 and T4 [15].

In addition to the follicular cells, the thyroid gland contains another endocrine cell: calcitonin producing C cells (parafollicular cells), partaking in the regulation of calcium balance in the blood circulation. These cells are also derivatives of the endodermal lineage, however not a part of the actual follicular unit, and give rise to the distinct cancer form called medullary thyroid cancer (MTC). These cells have not been studied in this thesis, nor the associated cancer form.

1.2.2 Taking a closer look at the thyroid follicular cell

The follicular architecture of the thyroid gland highly reflects the cellular function. The apico-basal polarity of the epithelium not only distinguishes the follicular lumen from extrafollicular space, but also the location of specific membrane bound receptors. At the basolateral membrane, the seven-transmembrane G-protein-coupled receptor for TSH (TSHR), the mono carboxyl transporter-8 (MCT8) of T3/T4 and the sodium-iodide symporter (NIS) are located. The apical surface harbors the iodide transporter pendrin, the membrane bound thyroperoxidase (TPO) and dual oxidase 1 and 2 (DUOX1/2). *(Fig.2)*

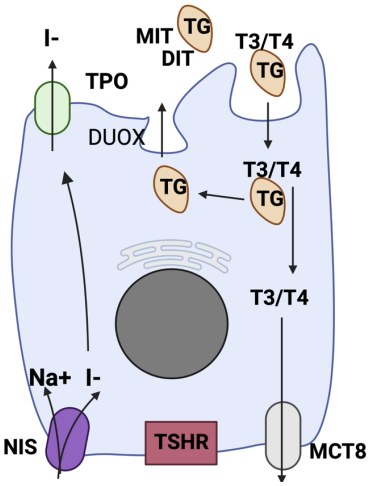

Fig.2. A (simplified) thyroid follicular cell/thyrocyte.

Thyroid hormones T3/T4 are made of thyroglobulin (TG) - a glycoprotein synthesized by the follicular cells and transported into the lumen via exocytosis - and iodide. The sodium-iodide symporter (NIS), encoded by the gene *SLC5A5*, enables active transport of circulating iodide into the follicular cell [16]. Further entry across the apical membrane to the colloid lumen is mediated via the transporter pendrin. Upon iodide entry, TPO will catalyze the oxidization and binding of one or two iodides to tyrosine residues on TG, in the presence of H_2O_2 produced by DUOX1/2, creating monoiodothyrosine (MIT) and diodothyrosine (DIT). Subsequent coupling of iodotyrosines creates either T3 (triiodothyronine) or T4 (thyroxin; tetraiodothyronine). Still bound to TG, T3 and T4 are transported into the follicular cell by endocytosis, where TG is subjected to proteolysis and excess iodide is recycled, leaving the hormones ready for transport into the circulation via the MCT8 transporter [17].

1.2.3 TSH regulation and natural follicle heterogeneity

Same, same, but different. The morphological and functional units of the thyroid gland are, as previously described, the follicles. Each one has the same basic architecture and components, and each one is surrounded by a fine capillary network. The homogenous distribution of vessels offers equal delivery of circulating TSH to evenly distributed TSHR at the basolateral membrane of the follicle cells, eventually triggering synthesis of thyroid hormones. However, the individual follicles and follicle cells are not entirely identical which is obvious already by looking at them in a light microscope (*Fig.3*).

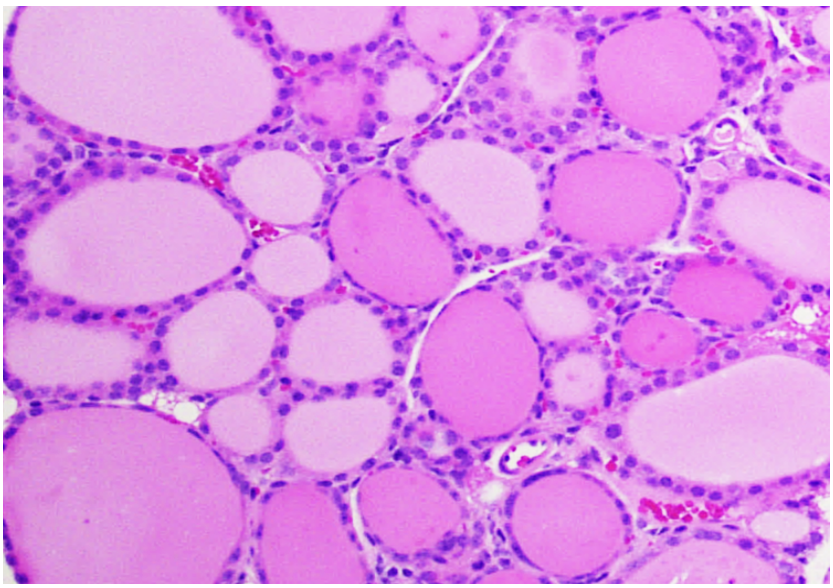

Fig.3. Micrograph of mouse thyroid follicles: thyrocytes with purple nuclei surrounding pink colloid filled lumina. Vessels with heavy stained pink erythrocytes are seen interspersed between follicles.

As previously described, thyroid hormone synthesis is regulated by the HPT axis. Upon peripheral hormonal demand, TSH from the anterior pituitary is released into the bloodstream. In humans and mice, TSH binding to the TSHR will initiate the cyclic AMP/protein kinase A (PKA) signaling pathway [18]. This will in turn stimulate the expression of several thyroid functional genes coding NIS, TPO and TG and regulate all the events subsequently leading to hormone synthesis and release. TSH has no mitogenic role during embryonic thyroid growth in mice, but postnatally it promotes reactive - benign - thyroid growth mainly due to proliferation of the follicular cells [18, 19]. It has been shown in mice, that excessive TSH stimulation causes thyroid hyperplasia

[20]. In thyroid cancer mouse models with mutant BRAF, dedifferentiation of thyroid specific genes leads to hypothyroidism, with subsequent elevated TSH that causes even more proliferation of the follicular cells and increased thyroid growth [21-23]

Already in the 1980's, individual follicular cells were shown to alter their iodination abilities with regards to factors like age and TSH availability [24]. The activity of TSH is reflected by the thyroid follicular histology. A stimulated follicle displays an epithelium that is nearly cuboidal with a reduced colloid content, whereas inactive follicles have large colloid-filled lumens - due to the retention of TG - and a flattened epithelium. Excessive stimulation by TSH in humans, due to low levels of free peripheral T3/T4, will both increase the number of thyroid follicles and their size, resulting in an enlarged thyroid gland - a goiter.

TSH levels affect the rate of endocytosis of TG from the follicular lumen, where a gradual increase of TSH stimulation causes a subsequent increase of endocytosis of TG. This functional intercellular heterogeneity can be identified already in early stages of thyroid development, since there are cells expressing TG even before organogenesis is complete [25].

The follicular structure, colloid content and TG expression was evaluated histologically as indicators of biological activity level and functionality of the epithelium in individual follicles in all studies of this thesis.

1.2.4 Thyroid development, folliculogenesis and functional differentiation

At the level of the 2nd pharyngeal arch, where the future tongue develops, the pharyngeal endoderm contains midline progenitor cells that upon the expression of four key transcription factors – Nkx2-1, Pax8, FoxE1 and HHex – will initiate thyroid development by specification of the endoderm and subsequently the formation of an outgrowth known as the thyroid bud. This developing bud – also referred to as the midline primordium – will eventually detach from the pharyngeal endoderm and move downwards to its final position ventral of the trachea, close to the developing heart and aorta and cranial of the budding future lungs, by the 7th week in humans and at E12.5 in mice [6, 26, 27].

The midline primordium is the major contributor of the thyroid gland and houses most of the follicular cells. In addition, the two lateral ultimobranchial bodies (UB), originating in the 4th pharyngeal pouches, will bring precursors of calcitonin producing C-cells. Previous consensus stated that the C-cells were derivatives of the neural crest and deriving from another germ layer, but it was recently shown by lineage tracing that these cells are also descendants of the endodermal lineage [28]. The midline thyroid grows, when in place, bilaterally along the 3rd pharyngeal arch artery and eventually fuses with the UBs, forming the final gland [26].

Directly after the fusion of the thyroid primordia, thyroid parenchyme will extend laterally in a branchlike pattern dependent on Fgf10 signaling [29]. The principle of branching morphogenesis is shared between several organs, the most obvious is probably the lung [30]. The final differentiation stages of the thyroid are *de novo* folliculogenesis - the assembly of polarized follicular cells and the formation of follicle lumina - and initiation of thyroid specific functional gene expression [27]. These events have long been considered occurring in a chronological manner, but a recent study by Johansson et al. [25] proves that the functional differentiation of thyroid cells and generation of follicles are in fact taking place at the same time but independent of each other. Furthermore, this is also the thyroid developmental stage with the highest proliferation rate.

The expression of TG is a completely unique feature of differentiated thyroid follicular cells, which is why we used the *Tg* promoter as Cre-driver to specifically target these cells with *BRAF*-mutation in the PTC mouse model used in this thesis.

1.3 THE LUNG

1.3.1 Take a deep breath - lung anatomy and function

Lungs have the function of oxygenating the blood via inspirated air brought into the close vicinity of carbon dioxide saturated blood in pulmonary capillaries. The lungs are placed in the thorax, bilaterally of the heart, and surrounded by a serous membrane called the pleura, allowing movement with low friction during respiration. The lung apices (superior aspects) extend into the neck above the 1st rib, while the basal aspects of the lungs rest on the diaphragm. In humans, the right lung has three lobes, separated by oblique and horizontal fissures, while the left lung has two lobes. In mice, the left lung has only one lobe, while the right one has four (*Fig.4*).

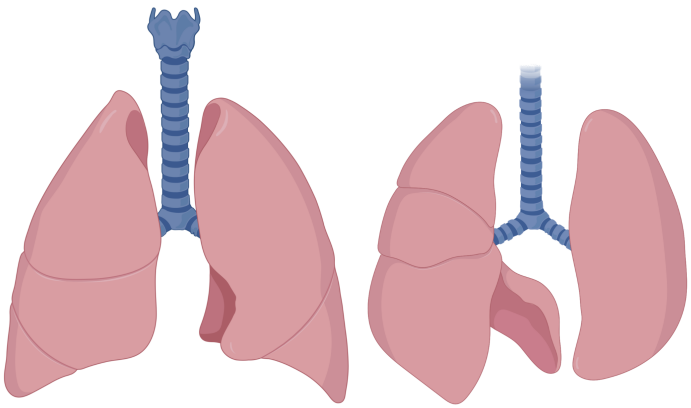

Fig 4. Frontal view of human lungs to the left and mouse lungs to the right

The air-conducting tracheobronchial tree begins at the larynx with the trachea in both species. At the sternal angle, the trachea bifurcates into two main bronchi entering, respectively, the right and left lung. The main bronchi divide into secondary lobar bronchi - three on the right side and two on the left side in humans; in mice four on the right side and one on the left [31]. Subsequently smaller and thinner branches develop, although in mice the final respiratory bronchioles are lacking, and the terminal bronchiole directly passes into the alveolar ducts. From the alveolar ducts the pulmonary alveoli - the functional and structural gas exchange unit - extends like small blister-like bunches [32-34]. The alveoli are surrounded by a fine capillary network consisting of endothelial cells resting on a basement membrane. Alveolar or pulmonary

macrophages are highly abundant, keeping the alveolar space free from debris and microorganisms.

Although the lungs harbor almost 60 different cell types [35], the tracheobronchial tree is lined by a pseudostratified respiratory epithelium mainly composed of three principal cell types. The larger conducts contain ciliated cells that sweeps mucus towards the larynx, goblet cells; producing mucus, and basal cells; regenerative cells replacing the epithelium. More distally in the bronchioles, the epithelium is of a simple columnar type and the goblet cells are replaced by secretory club cells (formerly known as Clara cells) [36]. Pulmonary neuroendocrine cells (PNEC), who detect hypoxia via chemoreception, are also scattered along the tracheobronchial tree. The final alveolar space contains two specialized epithelial cell types: type I pneumocyte - forming the alveolar lining, and type II pneumocyte - producing surfactant, a phospholipid that reduces the surface tension in the alveoli *(Fig.5)*.

In paper II we used antibodies against Club cell secretory protein 10 (CC10) and surfactant protein C (SPC) in lung tumors to identify the tumor cell-of-origin in the *BRAF*-mutated mouse model of lung and the dominating tumor cell type.

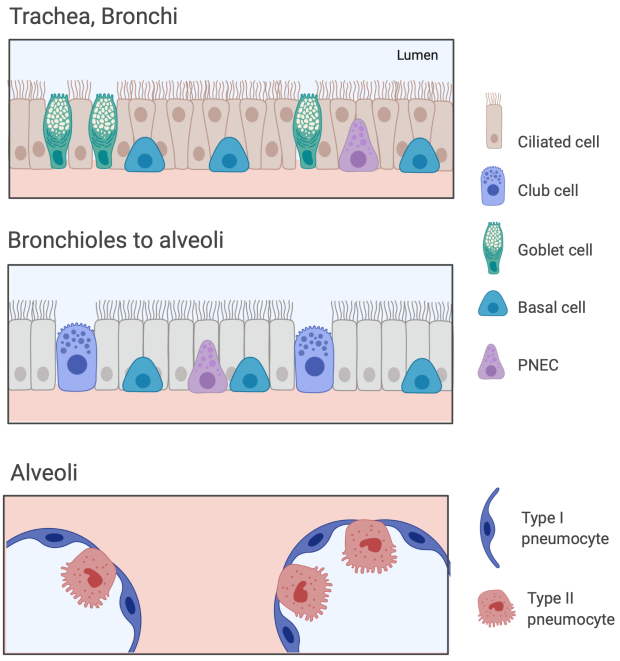

Fig. 5. Lung epithelium.

1.3.2 Lung development

Like the thyroid gland, the lungs are also endoderm-derived organs. The prospective respiratory system is first discerned in the beginning of the human 4th (E9.5 in mice) gestational week as a small ventral bud of the endodermal foregut. At the end of this week, the bud expands and undergoes dichotomous branching into the right and left primary lung and bronchial buds. After this, a sequential branching program is initiated, first forming the secondary bronchial buds (in humans three on the right side and two on the left, in mice four on the right side and one on the left), then all the structures of the bronchial tree [30], the lung parenchyme and finally the mature, functional alveoli [37].

When the primary lung bud first emerges, it protrudes into the mesenchyme (future muscular and cartilaginous supporting tissue and visceral pleura) surrounding the foregut, which will accompany the future budding-branching bronchi and regulate the branching pattern of the endoderm. This is accomplished by signaling of mesenchyme secreted fibroblast growth factor 10 (Fgf10) via Fgfr2b and regulated by negative signals from epithelial sonic hedgehog (Shh). Mice deficient of either of these factors will not develop lungs beyond the primary bud [38, 39].

The interactions of Fgf10 and Shh signaling is crucial during the development of several organs that involves branching morphogenetic programs, apart from the lungs including the pancreas and the thyroid. In *Fgf10*$^{-/-}$ embryos, thyroid branching and folliculogenesis is impaired, rendering a hypoplastic (yet still present) thyroid gland [29]. The expression of *Shh* is not present in the endodermal thyroid primordium region [40] and mice deficient of *Shh* will initiate thyroid budding, but the gland will not reach its final bilobed shape.

Almost in parallel with branching morphogenesis, distinct cell lineages begin to develop along the proximal-distal axis, differentiating endodermal lung progenitor cells into specialized ciliated and secretory (Club) epithelial cells, neuroendocrine cells and the alveolar type I and II cells (pneumocytes) [41]. These processes are regulated by the thyroid and lung mutual transcription factor Nkx2-1, which will be further described in the following chapter.

1.4 FOCUSING ON SOME IMPORTANT GENES

The morphogenetic events of the thyroid and lung are highly complex, and even though they are characterized in various animal studies, the molecular mechanisms are still undergoing investigation and more factors involved are continuously discovered [42]. The four transcription factors Nkx2.1, Pax8, Foxe1 and Hhex are proven highly essential for the thyroid follicular lineage and their combined expression is not seen elsewhere. Nkx2.1 is highly important also for the differentiation of lung progenitors. FoxE1 and Hhex are not in focus of this thesis, therefore only briefly described. In addition to these factors, although not investigated, the genes Foxa1 and Foxa2 are presented, since they are important factors in regional specification of the endoderm and development of the thyroid and the lung.

1.4.1 Nkx2.1 - a centerpiece in thyroid and lung epithelium

The homeodomain nuclear transcription factor Nkx2.1, also known as thyroid transcription factor 1 (TTF-1), is expressed in the thyroid, trachea, lungs, and certain areas of the brain. It is a hallmark transcription factor for the proper development and function of these organs. Indeed, human *NKX2-1* mutations may cause "brain-lung-thyroid" syndrome: chorea, infant respiratory distress, and hypothyroidism [43, 44].

In all papers included in this thesis, we used NKX2-1 as a biomarker in immunohistochemistry to identify epithelial tumor cell-of-origin, and in gene expression analysis for evaluation of the degree of functional dedifferentiation in response to mutant BRAF. In paper II, we also investigated the effects of conditional $BRAF^{V600E}$-mutation in thyroid and lung using *Nkx2.1Cre* and $Nkx2.1CreER^{T2}$ mice expressing tamoxifen-inducible Cre from the endogenous promoter elements of the *Nkx2.1* gene. It is therefore appropriate to give some further background information on the expression pattern and functions of Nkx2.1 in the thyroid and lung lineages.

$Nkx2.1^{-/-}$ mice will not survive beyond birth due to dysgenesis of the lungs with defective branching, nor will they develop a thyroid, although the midline primordium is initially formed [45]. Mice with heterozygous deletion of the *Nkx2.1* gene were previously reported born without an obvious phenotype [45], indicating that one allele is sufficient for normal development. This observation has been overthrown through several *in vivo* studies with $Nkx2.1^{+/-}$

mutated mice displaying different and milder thyroid and lung phenotypes than observed in *Nkx2.1* null mice [28, 46-48]. The different phenotypes are possibly due to pleiotropic functions of Nkx2.1 during development, depending on transcriptionally active domain, phosphorylation state and post-translational modifications [49],

Nkx2.1 expression begins just after the endodermal specification of the prospective thyroid and lung domains, around embryonic day (E)9.0 in mice and during the 4th gestational week in humans [50]. In the thyroid, NKX2-1 is ubiquitously expressed in the entire epithelial lineage throughout all developmental stages [29] and into adulthood, where it exerts regulation of thyroid specific genes encoding e.g. TG and TPO [27]. In the lung, NKX2-1 will, after initial budding, gradually display a gradient pattern with higher expression in distal airways. At birth, the expression is maintained in alveolar epithelium, mainly alveolar type II pneumocytes and club cells where Nkx2.1 regulates secretion of surfactant and club cell secreted protein, but it is decreased in other cell types [51, 52].

The relevance of Nkx2.1 expression in lung and thyroid cancer is non-arguable [53] due to its high tissue specificity. In clinical pathology and experimentally, it is used as biomarker to distinguish primary lung adenocarcinoma and thyroid carcinoma from metastatic tumors, to identify and assess differentiation state in relevant epithelial (tumor) cells and to determine tumor cell lineage [54, 55].

There is divergency regarding the impact of Nkx2.1 expression related to overall survival in patients with adenocarcinoma. Contradicting studies on human non-small cell lung carcinomas (NSCLCs) show correlation of NKX2-1 overexpression with both poorer and better prognosis [56, 57]. In one study, genomic profiling and functional studies of 52 lung adenocarcinoma derived cell lines and 76 tumors revealed DNA amplification spanning the *NKX2-1* locus and overexpression on both mRNA and protein levels [58]. Small interfering RNA (siRNA) mediated *NKX2-1* knockdown in the lung cancer cell lines caused decrease in cell proliferation and increase of apoptosis, and the authors stated *NKX2-1* as an implicated lineage-specific oncogene. The actions of NKX2-1 can thus be either tumor suppressing or tumor promoting, at least in the development of lung tumors, probably depending on cell state [59, 60].

In thyroid cancer NKX2-1 expression is shown to decrease in correlation with tumor progression [61]. Furthermore, decreased or absent NKX2-1 and PAX8 expression in thyroid tumors is associated with down-regulation of thyroid specific genes encoding TG, TSHR and TPO [62]. The clinical relevance lies

in the fact that in patients with poorly differentiated and dedifferentiated thyroid cancer, standard treatment with radioiodine is unsuccessful [63]. It was shown in a study using a patient derived cell line of poorly differentiated thyroid cancer, that transfer of Nkx2.1 into the cancer cells increased their uptake of iodide [64]. In paper II of this thesis, we specifically targeted Nkx2.1 expressing cells in thyroid and lung tissue with mutant BRAF.

1.4.2 Pax8 - keeping it together

The paired box *(Pax)* gene family consists of nine members, all sharing a common DNA-binding domain which is highly conserved between several species including humans and mice [65, 66]. All members are transcription regulating factors displaying various expression patterns in tissue derivatives of all embryonic germ layers. Pax8 especially is crucial for the development and function of thyroid follicular cells, excretory system of the kidneys, Mullerian tract and certain parts of the brain [67, 68].

As previously said, the coordinated gene regulation by the four transcription factors Nkx2.1, Pax8, FoxE1 and Hhex is needed to obtain and maintain a fully functional thyroid gland. *Pax8*$^{-/-}$ mice are born alive but severely hypothyroid due to regression of the midline thyroid, harboring most of the follicular cells, and die within 2-3 weeks [69, 70]. Studies have shown that Pax8 has an important role regarding cell survival of thyroid progenitor cells by blocking apoptosis [4, 70] in mice, which is also confirmed in humans carrying an inactivating *PAX* mutation, born with thyroid hypoplasia [71]. *Pax8*$^{-/-}$ mice may survive if administered substitutional therapy with T4, however they cannot bear offspring due to underdeveloped reproductive organs [72].

Apart from its fundamental role during thyroid gland development, Pax8 is important in the adult gland for 1) maintaining proper thyroid size by regulating apoptosis and 2) the functional differentiation of the follicular cells by regulating the expression of thyroid specific genes. In a study by Marotta et al, the conditional knockout of *Pax8* caused reduced expression genes regulating hormone production; *TSHR*, *Slc5a5* and *TPO* genes, but also *FoxE1* and *Nkx2.1* levels were lower [73].

The fact that Pax8 is normally expressed in only few distinct organs and in their progenitors, makes it a possible biomarker in tumor diagnostics of the thyroid, kidney and Mullerian system. PAX8 protein is indeed expressed in nearly all cases of well differentiated PTC and FTC [54, 74]. Unlike Nkx2.1,

Pax8 is not expressed in normal lung tissue or lung adenocarcinomas, which is why co-staining of these two factors may be used to distinguish the primary tumor ($NKX2\text{-}1^{+}/PAX8^{-}$) from a thyroid metastasis ($NKX2\text{-}1^{+}/PAX8^{+}$) [75].

In paper III and IV we used Pax8 as a biomarker to determine degree of differentiation of thyroid tumor cells by gene expression analysis and immunohistochemistry. We also co-stained lung tumors in paper II with NKX2-1 and PAX8, confirming them as primary.

1.4.3 Foxe1 and Hhex

Foxe1, formerly known as thyroid transcription factor 2 (TTF-2), is a member of the forkhead box family and like the FoxA-members a pioneer factor (described in the following chapter). Implied by its original name, this factor is expressed in the thyroid primordium, but also has a broad expression throughout the entire foregut endoderm [76]. *Foxe1*-null mice die shortly after birth displaying a cleft palate [77], probably causing severe feeding impairment. The thyroid phenotype of these mice is either an absent thyroid gland, or a sublingually positioned thyroid remnant. FoxE1 in the prospective thyroid is suggested as a downstream target of Nkx2.1 and Pax8, regulating migration of thyroid precursor cells after budding [70], since reintroduction of wild type *Foxe1* in such mutated cells will restore the gland position [26]. In human cancer, *FOXE1* is a confirmed susceptibility gene of non-medullary thyroid cancer [53, 78] and has also been shown to have effects during thyroid cancer development in mice [79]. It may also be of relevance in lung cancer development [80]

Hhex (hematopoietically expressed homeobox gene) is, like *Foxe1*, widely expressed in the foregut endoderm, eventually concentrated in regions including the prospective thyroid, lungs, pancreas, and liver [81]. $Hhex^{-/-}$ mouse embryos will not survive beyond E15.5, due to several organ malformations [82]. The initial specification of the thyroid primordium occurs, but soon thereafter the other three cornerstone transcription factors Nkx2.1, Pax8 and Foxe1 are downregulated. The subsequent events in thyroid development will not be completed, both suggesting individual relevance of Hhex as an expression maintainer of the other three factors, as well as their cooperative importance [70].

1.4.4 Members of the Fox family

The forkhead box (*Fox*) family consists in mammals of 44 subclassified genes, all sharing a distinct DNA-binding domain, broadly expressed during embryonic development and postnatally regulating various biological functions [83]. Hence, abnormal regulation of *Fox* genes has great impact on both developmental disorders and diseases.

During gastrulation, the FoxA family (Foxa1, Foxa2 and Foxa3) begin their expression. They are so-called pioneer factors, who facilitate chromatin opening and hence access to the genome for other transcription factors to bind to their specific sites. Both Foxa1 and Foxa2 establishes and maintains cellular identity in various endodermal-derived tissues e.g., thyroid, lung, pancreas, and liver. Foxa1 has a broader distribution of expression in adult tissue, but Foxa2 is the first factor expressed, which is reflected by the fact that *Foxa2*$^{-/-}$ mice have a much more severe phenotype and suffer from embryonic lethality. Foxa3 has the most restricted expression, mainly maintained in the liver.

Foxa1 and Foxa2 together take part in the foregut endoderm specification of the prospective lung primordium and will continue their expression throughout organogenesis and in pulmonary epithelium into adult age. Their critical cooperative role in lung formation and maturation has been proven by embryonic studies of *Foxa1*$^{-/-}$*/Foxa2*$^{+/-}$ mice, where severe branching defects and lack of epithelial differentiation is evident [84]. Conditional deletion of *Foxa2* alone disrupts normal development of alveoli but has no impact on branching morphogenesis. Interestingly, *Foxa1*$^{-/-}$ mice also do not have any altered phenotype regarding branching, indicating redundancy.

With regards to thyroid development, the expression of Foxa2 in the entire pharyngeal endoderm is non-arguable until the specification of the thyroid primordium, when Foxa2-expression drops in the region of the prospective thyroid bud and UBs [4]. Upon fusion of the respective anlagen, UB cells re-express not only Foxa2 but also Foxa1 and later display also co-expression with calcitonin [28].

1.5 THE CANCER CHAPTER

In this thesis we investigate tumor development and progression due to mutant BRAF in the thyroid and the lung, by conditional targeting of thyroid and lung epithelium in genetically engineered mice. In this chapter, thyroid cancer in general and papillary thyroid cancer in particular will first be described and to a greater extent than lung cancer, since the most of my scientific work is dedicated to the thyroid gland. But first I will introduce you to the most important contributors to our scientific work - the mice.

1.5.1 Mouse models of cancer

In vivo mouse tumor modeling has been a very valuable tool in cancer research for many years. There is a high level of genetic homology between mice and men [85], with many similarities in biology and normal development that reflects also on tumorigenesis. This, and the fact that mice are easily housed and bred having a short gestational period and rather short lifespan, make them a very good model system.

There are three main groups of mouse cancer models:

- Xenograft models (not included in this thesis); human cancer cells or tumor biopsies/fragments are transplanted into (more or less) immunocompromised mice, either ectopically or orthotopically - allowing e.g., in vivo studies of tumor cells and drug testing [86]. A possible disadvantage of such models is that immunosuppression may undermine studies of the cancer microenvironment [87]
- Models of induced carcinogenesis by chemical, viral or ionizing radiation agents (not included in this thesis) [88, 89]
- Genetically engineered models (GEMs). Genetically modified mice, enabling activation of (proto-)oncogenes or inactivation of tumor suppressor genes, described below in further detail.

The most straightforward way to reveal the function of a gene, is to abolish it and observe the consequences, i.e. knocking out the coding exon in embryonic stem cells [90]. A classic example is the mouse with a whole-body knockout of the tumor suppressor gene *Tp53*, developing various tumors [91]. In 1984, it was discovered that when injecting mouse oocytes with certain (onco)genes

the offspring was prone to develop tumors [92]. The pronuclear injections caused random integration of the exogenous gene sequence into the mouse genome leading to constitutive (over-)expression of the specific gene.

These conventional transgenic and knockout models revolutionized cancer research and studies utilizing them have provided immense knowledge about the impact of many oncogenes and tumor suppressors. However, since these genetic alterations are introduced already in the germline cells, all future cells are potentially and likely affected - resulting in major disadvantages like embryonic lethality and unexpected effects due to unpredicted genomic integration. These models are not well suited for modelling sporadic cancer development, but rather hereditary cancer syndromes due to germline mutations [93] .

The technology of introducing locus specific mutations into the genome, by injecting embryonic stem cells inside the blastocyst, enabled more targeted tuning of the expression of a single gene by introducing e.g. point mutations, deletions or translocations [94]. Mouse models that permit temporal and spatial specificity i.e., the possibility to target and regulate gene expression specifically within a certain tissue at a certain timepoint, are the conditional knockouts and knock-ins. This is the basic tumor model strategy used in all studies of this thesis which will be described in detail in the Methodology chapter.

1.5.2 Thyroid cancer

Among malignancies of endocrine organs, the most common is cancer of the thyroid gland [95]. The most prevalent subtypes are papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC), defined as well-differentiated carcinomas originating from thyroid follicular cells, but with differing characteristics clinically, histologically, and molecularly [96-99]. About 15 years ago, the entity of poorly differentiated thyroid cancer (PDTC) - representing an intermediate between well- and undifferentiated carcinoma with a worse prognosis than PTC and FTC - was introduced by the Turin consensus criteria [100, 101]. The most aggressive variant of thyroid cancer is anaplastic thyroid carcinoma (ATC) - an undifferentiated, non-curable cancer with very high mortality [102]. The fifth cancer form is medullary thyroid cancer (MTC), a separate entity arising from calcitonin producing parafollicular cells/C-cells situated in the interstitial space between follicles [103].

Worldwide, there has been an increase in thyroid cancer incidence generally and PTC especially during the past 30 years [104, 105]. Consensus state that the increase is relative; most likely due to higher frequency and quality of imaging and diagnostics, enabling identification of more small, asymptomatic tumors [106]. However, there are reports showing that other factors may have impact; more cases of larger PTCs are also being diagnosed, implying there is a true increase of incidence [107-109].

A proven environmental risk factor of thyroid cancer with emphasis on PTC is ionizing radiation exposure, especially in the pediatric population. The nuclear powerplant disaster in Chernobyl 1986 [110, 111] indeed caused a massive increase in the incidence of childhood onset PTC. One potential lifestyle related factor contributing to the increased incidence of PTC is obesity, especially in the USA [112]. Obesity may also be of prognostic relevance and has been shown associated with more aggressive PTC features [113].

1.5.3 Clinical presentation, diagnostics, and prognostic factors

The most classical presentation of a thyroid tumor is probably “a lump on the neck”, usually painless. Clinical symptoms are most often related to the neighboring tissues of the thyroid (tumor). Since the thyroid gland is in a sense “embracing” the trachea, a pronounced growth may cause slight compression of the same, wreaking difficulties in swallowing or breathing. Hoarseness, due to compression of the recurrent laryngeal nerves or enlarged lymph nodes are other symptoms. Imbalance of thyroid hormones, hypo-/hyperthyroidism is not a common debut sign. Rarely, in advanced cases, patients may present with symptoms related to distant metastasis in e.g., the lungs or bones. Also, thyroid tumors may be found incidentally in cases of imaging/radionuclide examinations of the head and neck region.

Upon suspicion of a thyroid tumor, after clinical examination, diagnostic ultrasound should be performed according to ATA guidelines [114] and EU-TIRADS [115]. Assessment of sonographic features is partly decisive for subsequent, selective, fine needle aspiration cytology (FNAC) scored according to the Bethesda system [116], assessing risk of malignancy. In most cases, malignant tumors are diagnosed by cytology alone, however, there are subtypes where morphology may be indeterminate and further analysis is indicated [117, 118].

If cytology is diagnostic for thyroid malignancy, next step is usually surgical removal of either one lobe or the entire thyroid gland, including suspected cervical lymph nodes, which will allow definitive histopathologic diagnosis [114]. Serum TSH levels are measured, evaluating thyroid function, and serving as a possibly negative prognostic factor [119-121]. To assess advanced disease - invasive, extrathyroidal growth, bulky tumor, lymph node engagement - additional imaging using computed tomography (CT), magnetic resonance (MR) or molecular imaging (PET, SPECT, Scintigraphy) may be indicated, providing important anatomical and additional information prior to surgery [122].

Prognostic factors like overall survival and risk of recurrence are highly related to the tumor itself. Proper TNM staging regarding size and extension (T), possible tumor spread to lymph nodes (N) and distant metastasis' presence and location (M) are therefore important [114], as is subtyping. Postoperatively, serum thyroglobulin levels are monitored as an indicator of recurrence or metastasis [123].

1.5.4 Subtyping of thyroid cancer

Traditional histopathologic criteria still serve as the foundation of diagnosis and subtyping of thyroid neoplasia. In addition, molecular analysis technologies and possibilities of detailed genetical characterization may identify distinct genetic aberrations that drive oncogenesis and/or tumor progression, enabling further subclassification of tumors. Many sub entities of thyroid cancer correspond to specific acquired mutations, most of them affecting the mitogen-activated protein kinase (MAPK) or RAS-RAF pathway *(Fig.6)* [124-127].

Normal BRAF and MAPK activity is regulated by negative feedback from downstream signaling kinases, but the oncogenic activity of $BRAF^{V600E}$ is both independent of extracellular factors and non-responsive to self-regulation (*Fig.6*) [128].

This year, the 5th edition of *WHO classification of endocrine and neuroendocrine tumors* is published, stating new categorization of thyroid tumors into benign, low-risk and malignant [27, 129]. In the malignant category, neoplasms originating from the follicular cells - distinguished by molecular profile and behavior - are classified as i) PTC: BRAF-like malignancies, encompassing several subtypes based on morphology;

ii) encapsulated follicular variant of PTC and FTC: RAS-like malignancies, more well-differentiated and less aggressive than PTC; iii) oncocytic[1] carcinoma (OCA); iv) high-grade[2] follicular-cell derived PDTC[3], and v) anaplastic thyroid carcinoma (ATC).

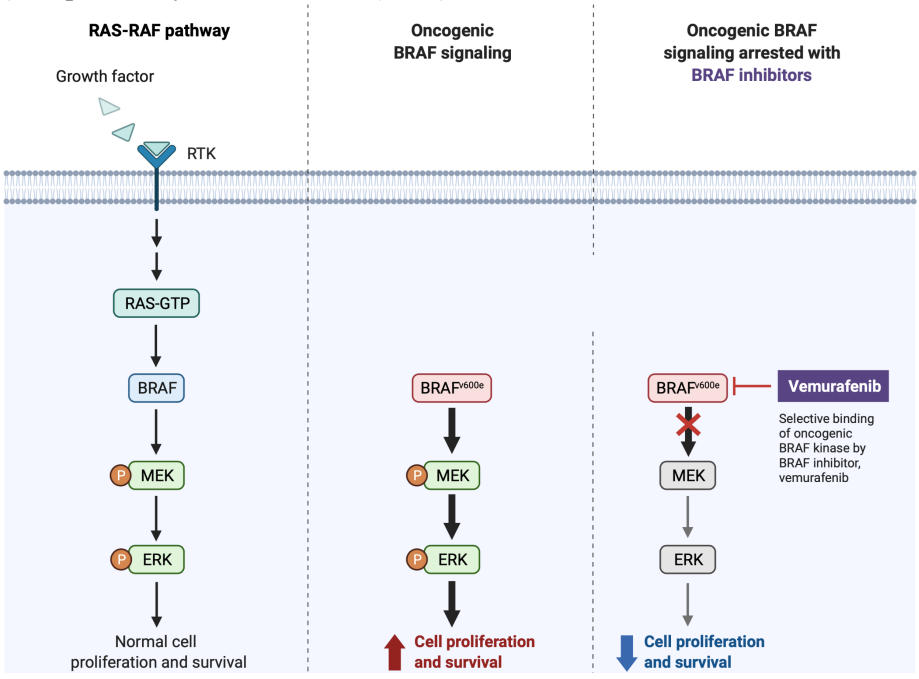

Fig. 6. Normal MAPK/RAS-RAF signaling (left), unrestrained mutant BRAF signaling (middle) and principle of BRAF kinase inhibition (right).

In PTC, the most common somatic mutation is of the *BRAF* gene, encoding oncoprotein $BRAF^{V600E}$, which causes an abnormal activation of the MAPK pathway and uncontrolled cell growth (*Fig.6)* [130-133]. This mutation is also found in cancer of the lung, skin (melanoma) and colon [134-136]. Mutations of the upstream signalling molecule of Ras, encoded by one of three different genes *HRAS*, *NRAS* and *KRAS*, are prevalent in FTC, follicular variant of PTC [137, 138] and in lung cancer [139]. Mutations affecting the cell surface receptor tyrosine kinase (RTK) may cause *RET/PTC*- rearrangements (classically induced by radiation) leading to oncogenic fusion proteins specific for PTC [140, 141].

[1] Displaying abundant eosinophilic granulae in the tumor cell cytoplasm.
[2] Invasive, containing necrotic areas and/or >3 mitoses per mm^2.
[3] Displaying insular, trabecular and/or solid growth without PTC nuclear features.

1.5.5 Papillary thyroid cancer

Papillary thyroid cancer (PTC) is the most common malignant thyroid neoplasm and accounts for more than 75% of thyroid malignancies [142-145]. It is also more common among individuals of female sex [146]. PTC is classified as a well-differentiated cancer with a 5-year survival rate of >95% [143], with a low mutational burden as shown in the Cancer Genome Atlas [147] and in most cases patients with PTC have an indolent disease with microcarcinomas[4] [142].

The entity PTC is originally named by the tumor growth pattern and specific nuclear features of the tumor cells. The tumors grow as papillae; fingerlike projections, with epithelial neoplastic cells surrounding a fibrovascular stroma (*Fig.7*) [96, 97]. The tumor cells have enlarged opaque nuclei ("ground-glass" or "orphan Annie"), nuclear foldings or grooves and occasional pseudo-inclusions [148, 149]. There are several PTC subtypes traditionally based on distinct features regarding their size (microcarcinomas), growth pattern (follicular, solid, micropapillary) and histological features of tumor cells, e g classical, tall cell, columnar cell, solid and hobnail variant. Morphological subtyping of PTC is prognostically important since some variants; columnar, tall cell, hobnail and solid are proven particularly aggressive although diagnostics have been extended for further subclassification [129, 150].

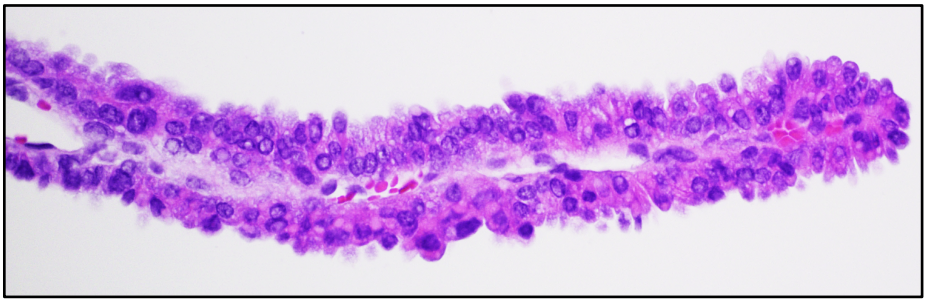

Fig. 7. Micrograph of a thyroid tumor papilla: crowded epithelial tumor cells are growing around a fibrovascular core. Tumor cells are pleiomorphic and display PTC nuclear features.

[4] Defined as less than 10 mm in diameter.

1.5.6 *BRAF p. V600E*

The spontaneous constitutively activating $BRAF^{V600E}$ mutation is an amino-acid substitution where valine (V) is substituted by glutamic acid (E). This leads to gain-of-function, abnormally high activity of the kinase domain and subsequent activation of MAPK, leading to uncontrolled proliferation, dedifferentiation and escaping apoptosis [151].

Mutant *BRAF* is the most common somatic genetic alteration and acts as oncogenic driver in the majority of sporadic PTC [99]. Although the overall prognosis of PTC is generally very good, $BRAF^{V600E}$ mutation is considered a negative prognostic factor. Advanced, aggressive PTC that displays lower degree of differentiation, invasiveness, recurrence, and resistance to treatment - hence with a less favorable prognosis - is more often BRAF-mutated [152, 153]. In fact, many cases of ATC harbor this mutation [154], and transgenic mouse models with combined $BRAF^{V600E}$-mutation and inactivated P53 show high degree of ATC development [155-157].

In recent years, an important factor in thyroid cancer progression, especially $BRAF^{V600E}$-mutated, is telomerase reverse transcriptase (TERT). It is a catalytic subunit of telomerase, adding tandem repeats of the sequence TTAGGG, telomeres, to the chromosomal ends, maintaining genome stability [158]. Telomere shortening is a normal event preventing excessive cell division when the telomeres reach a certain length [159]. In many cancers, reactivated telomerase thus enable cancer cells to become immortal [160]. There have been many studies characterizing *TERT* promoter mutations in thyroid cancer, revealing a consistent association with poor clinicopathological outcome [161-163].

1.5.7 Treatment of PTC - the crucial impact of NIS

Curatively intended surgery is the mainstay treatment of PTC and surgical radicality is undoubtedly a critical factor to minimize the risk of recurrence and metastatic spread [114, 164]. Second in line is the use of radioactive iodine (RAI) as a postoperative adjuvant, further described below. Additional postoperative treatment includes TSH suppression and thyroid hormone replacement (T4), conventional chemotherapy and/or external beam radiation therapy [114, 165].

The history of clinically implemented RAI started more than 80 years ago when Dr. Saul Hertz's experimental series on rabbits revealed that the thyroid gland was superior to all other tissues in the uptake of RAI [166]. The explanation of this phenomenon lies in the strong ability of the follicular cells to concentrate iodide, for which the sodium-iodide symporter (NIS) is a prerequisite [167, 168].

The first RAI treatment of humans was on hyperthyroid patients, with positive results [169, 170] and the first successful treatment of thyroid cancer, with metastatic spread, was reported in a landmark paper by Samuel Seidlin shortly thereafter [171]. Many years later, postoperative adjuvant RAI treatment with ^{131}I is often indicated and beneficial for ablation of post-surgery remaining tumor cells, metastases and in case of recurrence [114, 172, 173].

In most cases, surgery and RAI is sufficient to cure the patient, however, relapsing and progressing tumor growth with metastatic spread that is radioiodine-resistant accounts for 10-15% of cases with long-term poor prognosis [131, 155]. RAI refractoriness (RAIR) is suggested caused by a dedifferentiating effect of mutant Braf on thyroid follicular cells with down-regulation of genes encoding NIS, TG, TSHR and TPO [141, 174, 175]. On this basis, treatments targeting different members of the MAPK pathway have increased with the intention of re-sensitizing tumors to RAI.

1.5.8 Aiming at the target

In the past decade, the usage of tyrosine kinase inhibitors (TKIs) has become more frequent in thyroid cancer patients who are refractory to or progressing on standard treatment. Current TKIs approved by the FDA for treatment of advanced, radioactive iodine refractory thyroid cancer were recently reviewed by Lorusso et al and Cabanillas et al [176, 177]. These wide working agents have rendered some success; however, the drug effect is too often only transient, with considerable side-effects due to cross-reactions between various tyrosine kinases and acquired drug resistance.

The second generation of kinase inhibitors are more selective and aimed at the downstream targets of cell membrane bound receptor tyrosine kinases. To date, there are two FDA approved drugs, dabrafenib (selective inhibitor of $BRAF^{V600E}$) and trametinib (selective inhibitor of MEK*)* used in combination with indications ATC, melanoma and $BRAF^{V600E}$ mutated non-small cell lung cancer [178].

The selective $BRAF^{V600E}$ inhibitor vemurafenib (*Fig.5*) [179], FDA approved to treat *BRAF*-mutated melanoma, was the first used in *BRAF*-mutated thyroid cancer [180] with promising effects. In 2016, Brose et al reported results from a clinical phase 2 trial with vemurafenib antitumor effects in patients naïve to kinase inhibition and previously treated with a TKI [181].

The dedifferentiating effect of thyroid specific genes is also seen in mouse tumor models with thyroid specific expression of $BRAF^{V600E}$ [22, 23]. In this thesis, we used a similar mouse model pf PTC. In paper I, III and IV we used an analog (PLX4720) of vemurafenib to evaluate drug response, particularly the effect on thyroid specific genes including *Slc5a5* encoding NIS.

1.5.9 Sex disparities in papillary thyroid cancer

Clinically affecting differences between men and women are well-known in many cancers of non-reproductive organs [182]. Overall mortality rates, risk of invasive cancer diagnosis and incidence are generally higher in men; in both sexes the worst diagnosis in non-reproductive organs is lung cancer [183]. Many cancer types also show significant sex differences in response to therapy [184]

In differentiated thyroid cancer, FTC and PTC, the incidence and prevalence are nearly four times higher in women [183, 185, 186], but the survival rates and prognosis are still worse in males.

The female sex bias in PTC incidence, especially increasing at the onset of puberty [187], has raised the question whether estrogen (mainly E2) signaling might be an important factor [188]. Estrogen receptor (ER) expression in thyroid tissue was first reported in the beginning of the 1980's. All isoforms: Erα, ERβ and the membrane-associated, non-genomic signaling estrogen receptor GPER1/GPR30 have been detected in normal, benign, and malignant tissue/cells in both humans and rodents, mainly by immunohistochemistry and quantitative RT-PCR [189-191].

Experimental studies on thyroid cells have demonstrated that E2 generally has a highly stimulatory effect on cell growth by up-regulation of Erα [192]. Studies on human PTC and non-small cell lung cancer have indeed shown ERα as tumor promoting, while ERβ is considered to harbor a carcinogenic protective role, mediating e g pro-apoptotic signals [193, 194].

Existing mouse models of *BRAF* mutant PTC, with constitutive or induced expression of *BRAF*, do not show any overt sex differences regarding tumor characteristics or burden [23]. We suspected that synchronous targeting in most cells might mask subtle sex differences. To address the issue of sex bias in PTC, all experimental cohorts in paper I, III and IV were divided by sex.

1.5.10 Lung cancer

In paper II, we used a *BRAF*-induced mouse model targeting cells in both thyroid and lung. The mice developed progressive lung adenocarcinomas which are described in detail in paper II. A brief description of the lung cancer spectrum is provided here.

Very roughly, primary lung tumors are divided into carcinomas and carcinoids. Lung carcinomas are tumors of epithelial origin and the most common cause of cancer-related death worldwide [183, 195]. Carcinoids include tumors derived from mesenchymal and lymphoid tissue. The absolute majority of lung cancer patients have a history of cigarette smoking of different degrees, other carcinogens (by inhalation) identified are e.g., asbestos and radon.

Traditionally, lung carcinomas are divided into two main groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which includes adenocarcinomas, squamous and large cell carcinomas. WHO presented in 2015 a different classification [55], with more subgroups still based on traditional histology but also considering specific immunohistochemistry, resectability of the tumor, risk of metastasis and estimated response to conventional chemotherapy.

The major histologic types of lung carcinomas are adenocarcinomas (generally peripherally located tumors arising from glandular or secretory cells), squamous cell carcinoma (bronchial tumors with very close correlation to smoking history), small cell carcinoma (centrally located, necrotic tumors of neuroendocrine origin, highly metastasizing) and large cell carcinoma (a highly malignant, also neuroendocrine, tumor with different cellular phenotype from SCLC).

Regarding invasive adenocarcinoma, there are several subentities displaying differences in clinical symptoms, histological appearance, and growth pattern (mucinous/non-mucinous, acinar, papillary, lepidic, solid, stroma invading) imaging features and etiology [196, 197]. Mutations involving the MAPK pathway accounts for approximately ¼ of lung adenocarcinomas, and the majority affect the *KRAS* gene while *BRAF* mutations are rare (<5%) [198], although associated with more advanced disease, and like in PTC more women than men are carrying this mutation. The most common somatic mutation in lung cancer is, however, in the gene encoding the epidermal growth factor receptor (*EGFR*), a TRK that also exert signaling via RAS [199]

1.6 TUMOR CLONALITY - WHO'S TO BLAME?

A cell clone is formed by the progeny of one specific cell. In cancer, transformation of a normal cell (be it a progenitor or a differentiated cell) into a neoplastic one is traditionally described as a sequential chain of events, resulting in a tumor-initiating cell (TIC) or "the cell-of-origin", with its progeny thus constituting a tumor cell clone. This is not to be confused with cancer stem cells (CSCs), tumor propagating subpopulations of cells with the ability of self-renewal, deriving from the TIC [200, 201].

For PTC, the prevailing paradigm has been that tumors are originally monoclonal i.e., genetically homogeneous and deriving from only one TIC, based on of paired sequencing of primary tumors and metastases and genomic profiling from TCGA studies [147, 202]. However, these results disregards the fact that most (if not all) tumors display high levels of heterogeneity both inter- and intratumorally [203]. Several unsuccessful attempts with targeted monotherapies imply that monoclonality is perhaps more of a wish than a fact [204].

The concept of clonal evolution within tumor development and progression was stated by pathology professor Peter C. Nowell in 1976 [205]: *"it is proposed that most neoplasms arise from a single cell of origin, and tumor progression results from acquired genetic variability within the original clone allowing sequential selection of more aggressive sublines. Tumor cell populations are apparently more genetically unstable than normal cells. The acquired genetic instability and associated selection process, most readily recognized cytogenetically, results in advanced human malignancies being highly individual karyotypically and biologically. Hence, each person's cancer may require individual specific therapy, and even this may be thwarted by emergence of a genetically variant subline resistant to the treatment."*

According to clonality theory, during the process of tumor development, the progeny of the TIC may be subjected to further genetic events, affecting its genotype and (possibly) phenotype, thus generating "new" tumor clones of perhaps higher malignant potential (the multi-hit theory) [206].

Analyses of tumor cell subpopulations show high variability in the occurrence and frequency of mutations, genomic instability, epigenetic alterations etc. [204] However, all will probably not contribute to an actual phenotype. This means that not all mutations present in a tumor will have actual impact on its behavior unless certain premises. Most human tumors are caused by mutations

in oncogenes or tumor suppressor genes, where some mutations are “drivers”, but others are defined as “passengers” - only in it for the ride...

Driving mutations are just that, mutations that can initiate tumor development, start the process. But whether these (sole?) mutations are sufficient for progression is most likely dependent on the type of mutation, what developmental and differential stage the TIC is in when transformed and in which tissue the cell is residing, i.e. context-dependent [200, 207]. For example: *BRAF*^{V600E} mutation in melanoma and colon cancer is an initiating event but will not cause malignant disease unless additional genetic events occur [208, 209]. On the other hand, in thyroid and lung cancer BRAF^{V600E} alone may cause initiation and development (and progression?) of papillary carcinoma [23, 196]. In this thesis, we investigate how the developmental and differential stages of TICs affect tumor initiation, by stochastic activation of *BRAF*^{V600E} mutation, and development by using Cre drivers with different temporal onset during thyroid development. To address the issue of clonality, we performed lineage tracing using the double fluorescent reporter mTmG. In paper II, we also evaluate the impact of tissue-dependency by using a Cre driver expressed in two separate organs, thyroid and lung, deriving from the same embryonic germ layer.

1.7 TUMOR AND MICROENVIRONMENT - IT TAKES TWO TO TANGO

The normal purpose of inflammation is a defensive and healing process: it promotes the closing of a wound, eradication of invading microorganisms, reparation and regeneration of cells and tissue [210]. It is normally a self-limiting process, but when the stimulants are not eliminated, the inflammatory process may instead pose a possible threat.

More than 150 years ago, Rudolf Virchow identified leukocytes in tumorous tissues and suggested that cancer develops at sites of chronic inflammation [211]. It is now a known fact that inflammation serves as a cofactor during the development and progression of several cancers, whether it is caused by bacterial/viral/parasitic infection, extrinsic agents or autoimmunity [211]. In the last two decades, cancer research has shifted from the view of cancer as merely dependent on the tumor cells, to a more context-dependent, where the specific tumor surroundings are proven clinically relevant [212].

The tumor microenvironment is characterized by the present immunological cells; macrophages, lymphocytes, dendritic cells and mast cells, and the stromal compartment; connective tissue, extracellular matrix, and vasculature. In the inflammatory network soluble products are used as communicative and recruiting signals, the cytokines and chemokines, where some candidates may contribute to malignant progression [213]. In thyroid carcinomas, several studies have characterized the immunological cells and inflammatory components in manifest tumors [214] and there are proposals of including additional subtyping of thyroid cancer based on immune phenotype [215]. However, such studies are, as said, performed on manifest tumors, or patient-derived cell lines (from a manifest tumor) and do not provide information about preceding events in carcinogenesis that involves the immune system.

In vivo tumor models addressing such issues are lacking. Our PTC model (paper I) is based on spontaneously developing thyroid tumors within a normal microenvironment, which makes it suitable to investigate inflammatory contributions early in tumor development. In paper IV we investigated the spatiotemporal expression of three major proinflammatory cytokines involved in thyroid tumor progression [216]: tumor necrosis factor (TNF-a), interleukin 1-beta (Il1b) and interleukin-6 (Il6) and three chemokines: matrix metalloproteinase-2 (Mmp2), thrombospondin-1 (Ths1) and chemokine receptor type 4 (Cxcr4). We also investigated possible effects on cytokine expression after treatment with specific BRAF-inhibitor analog of vemurafenib (PLX4720).

MAJOR AIMS OF THESIS

Paper I: investigate whether spontaneous instead of induced activation of mutant *BRAF* in few targeted cells of *TgCreER^{T2};Braf$^{CA/+}$ mice* could mimic sporadic tumor development in the mouse thyroid gland.

Paper II: investigate the tumorigenic effects of constrained oncogene activation by leaky Cre activity in thyroid and lung tissues, using Nkx2.1 as Cre driver in *Nkx2.1CreER^{T2};Braf$^{CA/+}$* mutant mice

Paper III: elucidate whether the mouse model with stochastic oncogene activation in paper I might be instrumental to decipher the sex-bias in differentiated thyroid cancer observed clinically. Specific interest was paid to evaluate sex differences in response to targeted drug therapy with a BRAF-inhibitor.

Paper IV: characterize the time course of the inflammatory response to oncogene activation with focus on major pro-inflammatory cytokines and the generation of thyroid tumor heterogeneity.

2 METHODOLOGY

2.1 Conditional gene targeting by the Cre/loxP-system

In 1981, a bacterial recombinase was discovered by Sternberg and Hamilton [217] and got the name Cre (Causes REcombination). It is a site-specific recombinase that recognizes specific DNA-sequences (not present in mice) called loxP-sites, inserted on each side of a target genomic region, commonly referred to as a *floxed (flanked by loxP)* site. Upon recognition of the specific DNA sequences, Cre will excise the targeted region between them and conjoin, recombine, the ends (*Fig.8*).

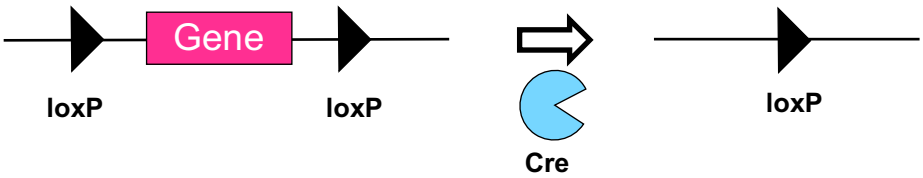

Fig. 8. Basic principle of the Cre/loxP system

Cre enzyme recombination between two loxP sites depends on their respective directions and will result in either the deletion or inversion of a gene, for example a tumor suppressor gene [218]. The floxed target sequence may also include an inserted STOP cassette. In that case, the excision and recombination will enable transcription of a downstream coding region previously inhibited and can be used to achieve temporal and spatial activation of an oncogene.

To obtain a conditional knockout or knockin mouse, two transgenic strains are crossed: one carrying the floxed target gene/genetic construct, without a phenotype and in theory equivalent to a wild type mouse. The genetic Braf construct used to generate $Braf^{CA}$ mice used in this thesis contains such a cassette [219], to ensure that the mutation is only activated upon Cre mediated recombination. The other mouse line carries the Cre recombinase under the control of a tissue-specific promoter, which means that Cre will not be expressed unless the promoter is active.

The pros of Cre-mediated conditional targeting are that normal gene function or mutations may be studied in specific organs/tissues/cells, without the confounding global effects occurring in conventional models. To date, there are numerous Cre drivers enabling tailored mouse models of a vast array of organs and cell types for studies of normal gene function and disease. Gene

targeting by the Cre/loxP-system is probably the most widely employed conditional mouse model [220, 221].

Many tissue specific genes are expressed during embryogenesis, and even though the Cre allows spatial control, early onset of a deletion or mutation may have grave impact on development and abolish the possibilities to investigate effects in the adult organism. To obtain not only spatial but also temporal control of gene expression, inducible Cre recombinases have been developed. In these models, Cre recombinase may be delivered by inhalation of Cre-carrying adeno- or lentiviruses [222], acquiring tight temporal control of Cre expression, but perhaps less selective spatially. In paper II we present a comparison between *BRAF*-induced lung adenocarcinoma induced by Cre inhalation and by

Another way of controlling Cre activity is by fusing a ligand-binding domain (LBD) to the Cre open reading frame [223]. The most common Cre-LBD fusion is the CreERT2-protein, with tamoxifen as the inducing agent [224]. The LBD is a mutated estrogen receptor that will not allow binding of endogenous estradiol. Without the inducing agent (tamoxifen), Cre remains in the cytoplasm due to the LBD binding heat shock proteins. The administration of tamoxifen will cause dissociation of the heat shock protein, enabling the CreERT2 to translocate into the nucleus and exert its effects on the floxed target gene.

In this thesis, we used three different Cre lines. One conditional Cre line under control of the *Nkx2.1* gene [225], to obtain tissue and cell specific expression of Cre in thyroid and lung from the onset of Nkx2.1 expression embryonically. To obtain a tighter temporal control of Cre, we used two $CreER^{T2}$ lines under control of the *Tg* promoter [226] and of endogenous promoter/enhancer elements of the *Nkx2.1* gene [227] . We crossed the Cre carrying mice with a mouse line carrying a genetic construct of a floxed $BRAF^{V600E}$ mutation with a preceding STOP cassette [219] to generate mice who develop sporadic BRAF-mutant PTC (paper I - IV) and lung adenocarcinoma (paper II).

2.1.1 Issues concerning Cre/loxP

No system is 100% flawless, the Cre/loxP is no exception. Sometimes, Cre is not activated in target cells despite ligand induced activation - probably due to different dose requirements of various tissues [228]. In other cases ectopic Cre expression in other tissues than expected, i.e. promoter leakage, or the inducing

agent per se may cause cellular toxicity [229]. Due to such occurring events, it is important to include proper controls when conducting animal experiments using Cre/loxP. In our mouse studies, we regularly used wild type and Cre carrying mice, where we ensured tamoxifen toxicity was not an issue and ruled out ectopic Cre expression by PCR on various tissues not expressing the Cre driving promoters.

Another conceptually different "adverse event" concerning Cre/loxP is Cre expression of ligand dependent Cre in the absence of the inducing agent (e.g. tamoxifen)[230], in theory from the onset of promoter (driver) expression, rendering stochastic genetic events in target cells rather than controlled temporal onset. This phenomenon has been regarded as disadvantageous in that it may lead to supposedly confounding effects; therefore, it is seldom documented or discussed and perhaps even ignored.

In the present thesis work, we looked upon this phenomenon differently and instead took advantage of spontaneous Cre-mediated recombination, modeling sporadic tumor development of the thyroid and lung in non-induced conditions.

2.2 Lineage and clonal tracing

In paper I and II we used the double-fluorescent reporter *Rosa*^{mTmG} [231] where all cells by default express a red fluorescent membrane protein: Tomato. Crossing the reporter mice with Cre carrying mice will enable, upon an active Cre, excision of the *Tomato* gene (*mT*) and its accompanying stop codon. This will allow expression of the green fluorescent protein GFP (mG), also membrane bound (*Fig.9 [231]*). After Cre has induced the red-green color switch in a certain cell, its progeny will also be labelled green, thus enabling cellular and clonal tracking.

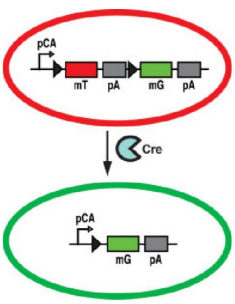

Fig. 9. mTmG reporter principle.

The intention for usage of the *mTmG* reporter was initially to quantify and trace cells co-expressing the reporter gene and mutant BRAF. However, in paper I we discovered that tumors consisted of either mT+ (red) cells, mG+ (green) cells or a mixed population. It was clear that many evidently mutated cells did not recombine the reporter gene. However, this phenomenon also revealed that many thyroid tumors were in fact of different clonal origin, described in detail

in paper I and further discussed in the Discussion chapter. In paper II, we observed similar events in both thyroid and lung tissue; in this study, it was evident that nearly all lung tumors were monoclonal and rarely displayed a mixed population of mT+ and mG+ cells.

2.3 Morphometry *ex vivo* or *in vivo* - the advantage of MRI

Measuring the total thyroid volume was a parameter used in all papers to evaluate tumor growth and possible drug effects on overall tumor size. In paper I and II we did this by measuring longitudinal and transverse (also used for depth estimation) diameter by a digital slide gauge and calculate the volume by the standard formula for ellipsoids $e=Height \times Width \times Depth \times (\pi/6)$ [232].

In paper I, III and IV we also used MRI as a means of noninvasive *in vivo* tumor monitoring. MRI is a highly versatile imaging tool, which can provide not only morphological information of high resolution but also enables quantitative measures of e.g. vascular perfusion and microscopic movement of water molecules, diffusion weighted imaging, exemplified in paper I, or metabolic activity [233]. We used T2 weighted MR-images to calculate tumor volumes from multislice anatomical MR-images [234] using a GUI written in MatLab_R2018b. Detailed image acquisition parameters are described in the respective papers.

The usage of MRI monitoring of tumor growth not only provided precise calculations of tumor volume and high-resolution images, but it also allowed us to follow individual animals over a long time, something that has never been done before in experimental studies of PTC. Another advantage with this technology is that it is an imaging technology not based on ionizing radiation, which is for one a known cause of DNA damage and in the case of thyroid cancer a possible confounding factor in studies of radiotherapy with ^{131}I [235] or other radionuclides. Further utility of MRI in our mouse tumor models will be discussed in the Future prospects chapter.

3 SUMMARY OF RESULTS

3.1 Paper I. Tissue architecture delineates field cancerization in *BRAF^{V600E}*-induced tumor development

In paper I, we investigated if sporadic PTC with focally activated mutant cells in a normal follicular environment is a more suitable model to monitor tumor growth in different stages.

By crossing *TgCreER^{T2};Braf$^{CA/+}$* mice with *mTmG* reporter mice, also without tamoxifen injection, occasional Cre activation was evident in a minority of thyroid follicular cells postnatally. The thyroid morphology at three months postpartum was generally normal, with occurrence of dilated follicles and focally hyperplastic epithelium. At 6-12 months, multicentric tumor foci with papillary growth, exhibiting different PTC morphology (classical, cystic, hobnail, tall-cell, and solid variants), were evident. Lineage tracing revealed that tumor initiating events started early, even before birth and that many subsequently hyperplastic follicles and manifest lesions were displaying a mixed population of mT+ and mG+ cells, indicating an oligoclonal origin.

There was a significant difference in thyroid (tumor) volume between female and male mice at six months, with females displaying larger tumors. Furthermore, after six months and onwards, differences in lobe size increased, emphasizing heterogeneous growth. Immunohistochemistry (IHC) revealed largely retained expression of NKX2-1 and E-cadherin, but with the latter often showing basal displacement of adherens junctions. Staining of TG showed complete loss of expression in manifest carcinomas. MRI with diffusion weighted imaging at 12 months in a sporadic *TgCreER^{T2};Braf$^{CA/+}$* mouse, showed lower diffusion in solid tumor areas.

Treatment with the BRAF kinase inhibitor, vemurafenib-analog PLX4720, from weaning (4 weeks age) until 3 months of age resulted in diminished tumor growth, although not to complete remission, indicating drug resistance in certain tumor cell clones.

3.2 Paper II: Stochastic targeting of *Nkx2.1*-lineage cells differentially recapitulates BRAF-driven tumor development and progression in lung and thyroid

In paper II we investigated stochastic activation of $BRAF^{V600E}$ by spontaneous Cre activity (omitting tamoxifen induction) using another promoter, *Nkx2.1*, which is a corporate gene expressed in precursors and differentiated cells within the thyroid and lung.

Conditional knock-in of mutant *BRAF* under the *Nkx2.1* promoter - generating $Nkx2.1Cre;Braf^{CA/+}$ mice - caused massive epithelial hyperplasia specifically in *Nkx2.1* lineage cells, in both embryonic thyroid and lung tissues. The abnormal lungs gravely impaired respiratory function, leading to early postnatal lethality. Since the conditional knock-in displayed such global effects in both the thyroid and the lungs, we employed the $Nkx2.1CreER^{T2}$-line - designed to both abolish the *Nkx2.1*-gene of one allele and recapitulate endogenous expression upon Cre expression - in attempt to recreate sporadic BRAF-driven tumorigenesis in thyroid and lung.

Using the same strategy as in $TgCreER^{T2};Braf^{CA/+}$ mutants in paper I, i.e. not inducing Cre activity with tamoxifen, we found that the percentage of spontaneously activated Braf mutant cells in the thyroid - analyzed by SiMSen-Seq technique [236] - was significantly lower in $Nkx2.1CreER^{T2};Braf^{CA/+}$ mice than in $TgCreER^{T2};Braf^{CA/+}$mice. The thyroids of $Nkx2.1CreER^{T2};Braf^{CA/+}$ mice were at the age of 6 months comparable to wild type thyroids; neoplastic lesions were few and restricted to single follicles.

The opposite effect was seen in the lungs of $Nkx2.1CreER^{T2};Braf^{CA/+}$ mice, where the percentage of spontaneously activated Braf mutant cells was higher than in the thyroid. Multiple, atypical hyperplasia and small adenomas scattered throughout the tissue were present already at the age of 1 month. All mice displayed gradual tumor development of adenocarcinomas, causing death due to respiratory failure around the age of 6 months. Pulmonary neoplastic lesions displayed diminished NKX2-1 expression at early stages, compared to surrounding alveolar type 2 cells, but also heterogeneity of expression within tumors. IHC analysis showed that predominantly tumor cells were positive for surfactant protein C (SPC) and negative for CC10 (biomarker of bronchiolar Club cells), confirming cell-of-origin as alveolar type 2 cells. Clonal tracing in non-induced $Nkx2.1CreER^{T2};Braf^{CA/+};mTmG$ mice revealed most lung tumors as monoclonal, with the majority not displaying any reporter activity.

3.3 Paper III: Sex bias of BRAF-inhibitor therapy in mice with papillary thyroid cancer

Clinical use of tyrosine kinase inhibitors is mainstay in the management of many types of malignant tumors. By targeting key components in crucial signaling cascades, the goal is to specifically target neoplastic cells with a certain driving aberration with as little side effects as possible [176]. In paper III, we used the sporadic PTC-model generated in paper I to investigate the effects of PLX4720, prodrug of the selective Braf-kinase inhibitor vemurafenib in *TgCreER^{T2};Braf $^{CA/+}$*mice. Current indications for treatment (FDA approved) with vemurafenib are unresectable or metastatic melanoma [237] and Erdheim-Chester disease [238] with BRAF^{V600E} mutation. Indications for treatment of metastatic and unresectable, radioiodine resistant BRAF^{V600E}-positive PTC is under evaluation.

Two experimental setups were used on *TgCreER^{T2};Braf$^{CA/+}$*mice, all experimental cohorts divided by sex:

1) continuous treatment with PLX4720 or control dietary pellets starting at 4 weeks of age, representing an early stage of tumor development, with monitoring of individual tumor growth by MRI up to 12 months of age. At study endpoint, thyroids were morphologically and histologically examined.

2) continuous treatment with PLX4720 or control dietary pellets starting at 6 months of age, representing a later stage in tumor development, with treatment for 4 weeks. Tumor volume was monitored and calculated using MRI before and after treatment and morphological/histological evaluation was performed at study endpoint. To evaluate effects of Braf-kinase inhibition on thyroid specific genes (*Nkx2.1, Pax8, TSHR*, *Slc5a5* (NIS)*, Tg, TPO*) gene expression analysis by real time quantitative PCR (qPCR) was performed.

Results of experimental setup 1 confirmed our initial findings in paper I: initial PLX4720-treatment response by reduced tumor growth in treated animals compared to untreated animals at 4 months of age, measured by MRI. At 12 months, the thyroids of PLX4720-treated and non-treated males were nearly equal in size, whereas the PLX4720-treated females displayed accelerated tumor growth. Morphologic examination revealed partial restitution of thyroid follicle architecture in the treated males, however papillary tumors were still present, while the females exhibited advanced, invasive tumors with signs of dedifferentiation in tumor cells.

Results of experimental setup 2: also in this scenario, males showed better response to treatment regarding tumor size (reduced) and histological appearance (restored follicular architecture). Analysis of thyroid specific genes by qPCR revealed striking sex differences in recovery of gene expression in *TSHR* and *Slc5a5* after treatment, with males showing better recovery. Staining for TG revealed normalization of protein expression in treated male thyroids, indicating functional thyroid follicular cells, whereas in females the TG expression was comparable to non-treated female mice.

3.4 Paper IV: Heterogeneity of *Braf*^{V600E}-induced cancer inflammation in a mouse model of sporadic thyroid tumorigenesis

The impact of inflammation and stromal derived factors in tumor progression has been studied in various (thyroid) cancer forms, but studies regarding the influence of cytokines and chemokines in early stages of carcinogenesis are lacking [213].

In paper IV, we used the PTC-model (paper I), *TgCreER*^{T2}*;Braf*$^{CA/+}$mice, for investigation of the presence and influence of pro-inflammatory mediators in early and later stages of thyroid tumor development. Cytokines Interleukin-1 beta (Il1b), Interleukin-6 (Il6) and Tumor necrosis factor-alpha (Tnfa), and chemokines matrix metalloproteinase 2 (Mmp2), thrombospondin-1 (Thbs1) and chemokine receptor type 4 (Cxcr4) all showed a gradual increase of gene expression between 1 and 6 months of age in non-induced mutant mice, and a subsequent decrease at 12 months. Interindividual variation of expression was high at all ages, as was the expression in isolated tumor foci investigated by qPCR at 12 months.

IHC analysis of the cytokines Il1b, Il6 and Tnfa revealed that the initial expression up to 3 months of age was derived from the neoplastic epithelial cells, whereas expression in stromal tissue and immune cells was scarce. The expression was heterogeneous both interindividual and within tumors of the same individual. At 6 months, there was a substantial increase of tumor surrounding stromal tissue, with several morphologically different cells expressing cytokines.

PLX4720-treatment for 4 weeks at the age of 6 months reduced the expression of proinflammatory genes in thyroids of *TgCreER*^{T2}*;Braf*$^{CA/+}$mice, measured by qPCR, compared to untreated *TgCreER*^{T2}*;Braf*$^{CA/+}$mice. IHC analysis of the cytokines Il1b, Il6 and Tnfa did not show any overt differences between PLX4720-treated and untreated mice.

4 DISCUSSION

The life of a tumor consists of three basic stages: initiation, development, and progression. If it was up to me, I would add a fourth stage - death - the earlier the better.

In cancer research the desire and aim is to dissect and understand the mechanisms behind the processes that causes a cell to abandon its pre-intended purpose, to transform, to ignore normal regulating signals and start to multiply, to become a tumor initiating cell (TIC). The next stage following initiation is the tumor development, when the progeny of the TIC procreates, forms a cellular clone, possibly including cancer stem cells, that expands and claims space on behalf of normal tissue and nourishments by surrounding circulation. Hopefully, this is where it all comes to an end. If we're lucky, intrinsic cellular mechanisms causes the tumor cells to stop dividing and possibly die, or the immune system brings in the cavalry and kills them, or we have detected them and provided successful (tumor cell killing) treatment. However, this is not always the case, since tumor cells are cunning little beings, highly adaptable and experts in taking advantage of situations potentially harmful to them. And so, we continue the struggle.

4.1 ReCREating BRAF-driven papillary thyroid cancer

For me, the whole idea of tumor modeling is to mirror, or mimic, the situation in the human organism. PTC in humans is considered a somewhat "simple" cancer, due to its low somatic mutational burden and in most cases a single driving mutation is enough to cause a tumor. Despite this, the PTC phenotype is highly diverse. In patients, many only have a microcarcinoma <1cm and most are cured by surgery alone, but some progress to highly advanced stages with metastatic disease and resistance to treatment. The heterogeneous phenotype is also seen in the microscope, with several histopathologic subgroups comprising cellular variants like tall cell, columnar cell, classical, hobnail and various growth patterns like follicular, papillary, sclerosing, solid… The reasons for this heterogenous phenotype are largely unknown, although we propose in paper I that the explanations lie in the natural heterogeneity and individual integrity of the thyroid follicles, in combination with the (age-related?) onset of oncogenic mutation [24, 207]. Moreover, humans with PTC rarely present with signs of hypo- or hypothyroidism, indicating hormonal levels as basically still normal, which is also seen

microscopically since the tumorous tissue is admixed with follicles of normal appearance, most likely functional.

The bearing concept throughout this thesis is about the way we use a genetically engineered mouse model. The model is based on conditional expression of mutant $BRAF^{V600E}$ in thyroid and lung using Cre/loxP mediated recombination (described in Methodology section). To obtain spatiotemporal control of oncogenic activation, we used tamoxifen inducible $CreER^{T2}$ [226] mouse lines and crossed them with a mouse line carrying floxed mutated $Braf^{CA}$[219]. We did this to generate a mouse model of sporadic papillary thyroid cancer (PTC), the most common human thyroid cancer, which in most cases is also BRAF-mutated.

In our first study, paper I, the tissue specific promoter gene driving Cre expression was *thyroglobulin (Tg)*, which is only expressed in differentiated thyroid follicular cells, with onset around embryonic day 15 in mice. After generating $TgCreER^{T2};Braf^{CA/+}$mice, we followed the protocol to activate the Cre enzyme and injected the mice with tamoxifen at 4 weeks of age. After 2 weeks we noted a swollen thyroid gland with altered morphology; enlarged follicles with occasional hyperplastic epithelium. After 2 months there was not a single normal follicle to be seen; the heavily enlarged gland displayed a completely deranged architecture with rather compact sheets of tumor cells and thick bundles of stroma interspersed. Hormonal measurements of these mice proved them to be severely hypothyroid. This is not mimicking human PTC.

In a control mouse, carrying $TgCreER^{T2}$ alone, my colleague Shawn Liang had previously performed X-gal staining on thyroid sections from both induced and non-induced $TgCreER^{T2}$ mice, to confirm tissue specificity of Cre. He observed that in the non-induced mouse, there were a few positively stained cells, confirming Cre activity without induction by tamoxifen, commonly referred to as “leaky Cre activity”, although a more proper description would be inducible Cre-dependent activation without induction (a bit confusing perhaps). The phenomenon had been observed before and considered a huge disadvantage, with the explanation that the intended spatiotemporal control of Cre expression had been compromised.

We decided to use the supposed disadvantage as an advantage. In $TgCreER^{T2};Braf^{CA/+}$ mice, we thus allowed spontaneous activation of Cre and oncogenic BRAF by omitting induction with tamoxifen. By doing this, we eventually observed dilated and neoplastic follicles which subsequently developed into multifocal papillary microcarcinomas in a stochastic manner at 6 months of age. There were many normal follicles in the tissue surrounding

the tumors, and hormonal analysis revealed the mice as euthyroid, thus deducing any growth stimulating effect by elevated TSH following low peripheral T4/T3. Over time, the microcarcinomas progressed into overt PTC displaying multiple histological phenotypes, some with aggressive infiltrative growth pattern into surrounding muscle tissue and signs of Epithelial-Mesenchymal-Transition by diminished expression of E-cadherin, particularly after 12 months of age. Not all neoplastic foci progressed into actual tumors, indicating they were either quiescent or subjected to oncogene induced senescence.

It is our solemn belief that this approach is a more accurate, although time-consuming, way to model human sporadic PTC.

4.2 Intended outcomes and surprising findings

To quantify the rate of sporadically *BRAF*-mutated thyroid follicular cells and clonally trace the progeny of these cells, we crossed the *TgCreER^{T2};Braf$^{CA/+}$* with the double fluorescent reporter mTmG (described in Methodology section). The hypothesis was that upon Cre recombination cells with an active *BRAF* mutation would also activate the reporter gene and switch color from red to green, thus the developing tumors would be easily distinguished and quantified. However, clonal tracing revealed that neoplastic follicles and developing microcarcinomas were not solely harboring green cells (mG+), but also red (mT+), in fact most of them failed to recombine the reporter gene and remained mT+ alone. The explanation of this phenomenon is probably that when recombination occurs stochastically, non-parallel recombination causes sequential activation of transgenes instead of simultaneous [239]. This means that in some cells the reporter gene was activated first, enabling the color switch before mutant *BRAF* was activated, and in other cells the scenario was opposite. However, this does not explain why most tumors remained mT+.

It is known that *BRAF* mutation and constitutive activation of the MAPK pathway rapidly causes downregulation of thyroid specific genes like Tg. Thus, if *BRAF* was firstly activated by recombination in one cell, there is a possibility, supported by our findings of predominantly mT+ tumors, that Tg was downregulated before Cre could activate the reporter gene. These results undermined our means to quantification of mutated cells; however, they led to very important observations. Within single follicles and subsequently derived tumors, there were clear evidence of multiple tumor clones - some cells were mT+ and other mG+.

The concepts of clonal expansion and multiclonal origin do not have to be mutually exclusive. In fact, they might just be the opposite. Thyroid follicles are polyclonal by nature, and the phenotypic diversity and heterogeneity of PTC is convincing. It is probable, as seen in our clonal tracing experiments, that individual tumor clones cooperate during tumor development, and that cell competition threshold mechanisms against normal surrounding cell populations determines whether the tumor will progress or not. Certain clonal cooperativity, for example between tumor cell clones and stromal clones is probably a prerequisite for metastatic spread or recurrence [240], possibly in combination with other stress factors. A more homogeneous cell population, as in the case of tamoxifen induction, will undermine the possibilities to study such mechanisms, since homogeneity infers non-mosaicism. This is another reason why we believe our approach, omitting induction, is a better way to address such questions.

There is probably a threshold to when a malignant cell or cell clone outcompetes the normal population and on the other hand when the normal population forces the malignant clone to succumb or move into dormancy. The mechanism(s) behind such a threshold could be influenced by both the timing of a mutation and in which tissue/cell. In paper I the Cre driver was *Tg*, whose expression coincides with a period of high proliferation and rapid cell cycling during thyroid development. In paper II we used *Nkx2.1*, whose expression is commenced during a period of low proliferation, as Cre driver. There was a very clear difference in thyroid tumor development using one or the other. In $TgCreER^{T2};Braf^{CA/+}$ mice signs of thyroid neoplasia was evident already at 1 month of age and they eventually developed large tumors. In $Nkx2.1CreER^{T2};Braf^{CA/+}$mice, there was no evident signs of neoplasia until between 3-6 months of age, and the lesions were very small and confined to a single follicle. On the other hand $Nkx2.1CreER^{T2};Braf^{CA/+}$mice instead developed lung adenomas evident at 1 month of age. Many of these adenomas progressed into large invasive adenocarcinomas. The results from paper II indicates that cell state is not the only factor influencing tumor development, but also the tissue related setting. The mechanism(s) behind this is yet to be investigated.

4.3 Finally - let's talk about sex

Women and men are equal, but to think we are the same is to fool oneself. It is not only the X or the Y that has potential impact on how much and in what ways we are affected by disease. The concept of gender, a social construct involving family context, economy, education, ethnicity, and many other factors may have more impact than we know. For example, men are less prone than women to seek medical care, and possibly more exposed to certain risk factors like smoking or drinking. On the other hand, women are more affected by stress-related disorders that may cause overall impact on quality of life. Such factors are relevant and should be considered when optimizing patient care.

With that said, the biological sex is most definitely relevant concerning clinical conditions like cancer. There are differences in susceptibility, incidence, prognosis, treatment response and mortality, long known factors that for a long time have been accepted without further investigation. This is beginning to change, by analysis of sex differences on the molecular level [241] and the landscape is vast [242]. Sexual dimorphism has been found in genes involving e.g. metabolism, hormonal signaling (non-reproductive) and immune system.

In paper I, we observed an obvious difference in tumor size between male and female mice, more by chance than intent, where the females developed larger tumors. These observations led to the study design in paper III, where all animal experimental groups were divided by sex. Initial observations were confirmed and expanded when we found that there were striking differences between the sex-based cohorts regarding drug response. These are interesting findings, since PTC has a higher incidence in women, but the men are more subjected to advanced disease and have a higher mortality risk. In our mouse model, the concept of gender is not applicable (as far as we know!), which is why we interpret the differences as truly biological. Although we do not know whether these differences may be extrapolated to human patients, it does shed light on another issue regarding basic research, which is to include the aspect of sex when performing animal experiments.

CONCLUSIONS

By omitting tamoxifen induction in *TgCreER^{T2};Braf$^{CA/+}$* mice we have generated a mouse model of sporadic tumor development and progression of papillary thyroid cancer (PTC), that faithfully recapitulates the situation in humans

Using clonal lineage tracing we have revealed that tumor initiation starts perinatally by oligoclonal growth of Braf mutant cells and that developing tumors exert clonal cooperativity. Tumorigenesis can be traced back to individual follicles which forms the basis of tumor heterogeneity.

In the PTC model, sex differences in tumor growth and drug response not previously reported in mouse tumor modelling has been brought to surface. Females develop larger tumors and show a poorer recovery response of silenced thyroid gene expression to BRAF-inhibition than male mutant mice. Moreover, long-term treatment with vemurafenib (PLX4720) aggravates tumor progression only in females.

Cytokine expression in the PTC model reflects tumor heterogeneity spatiotemporally with the same oncogenic Braf mutation. Tumor cells express cytokines in early stages, immune cell recruitment is a later event and vemurafenib treatment causes normalization of cytokine expression

Similar stochastic thyroid targeting of mutant Braf using *Nkx2.1* as Cre driver leads to constrained tumorigenesis depending on a low rate of *BRAF* activation. By contrast, the very same mice develop multiple lung adenocarcinomas predominantly derived from surfactant-producing alveolar type II pneumocytes. This represents a new mouse model of non-small cell lung cancer due to *BRAF* mutation.

FUTURE PERSPECTIVES

The non-induced mouse model of PTC has already proven to be useful in addressing various issues regarding tumor development and progression. We are planning experiments with RAI therapy in combination with the already tested Braf inhibitor vemurafenib, other Braf-inhibitors, other inhibitors of the MAPK-pathway and recombinant TSH. In a near future, we will have a unique opportunity of performing PET/MRI on our mice, which means we may perform accurate dosimetry planning of RAI and determine at what stage in tumor development the cancer cells are most susceptible to treatment.

On our doorstep is of now transcriptomic data of tumors from animals in paper III. We will analyze these data from the hypothesis that there might be sex-biased tumor identity. Another question we would like to answer is whether there are molecular biomarkers indicating good/bad response to Braf inhibition.

Based on findings in paper IV we will continue to map the impact of the immune system on tumor development and progression in sporadic PTC. We want to characterize the subpopulations of immune cells infiltrating early, intermediate, and late tumor stages. Furthermore, we plan to characterize the stromal contributions with emphasis on cancer-associated fibroblasts, since we have observed that the fibrous tumor stroma increases over time.

Based on our findings in paper II, we want to use our new mouse model of Braf-induced lung adenocarcinoma and perform various experiments involving further characterization of the biological mechanisms that causes an adenoma to switch into an adenocarcinoma. We plan to perform drug experiments with kinase inhibitors or other compounds relevant.

Regarding the rather discrete microcarcinomas observed in thyroids in paper II, we aim to investigate whether this model might be equivalent to the indolent thyroid tumors prevalent in humans. If we treat the animals with Braf inhibitor and pull the brakes on the lung tumors, we might be able to evaluate whether the thyroid lesions will progress into therapy resistant PTC or if they remain in the same state.

As a very intelligent person once wrote in a thesis: there is <u>still</u> a lot of work to be done!

ACKNOWLEDGEMENTS

To be a researcher is not a job, it's a lifestyle! There are so many people that has have been involved in different ways during the making of this thesis, who have contributed with knowledge, experience, tips and tricks, products, their own time, conversations, laughs, chocolate… I am so thankful for all of you!!

Mikael Nilsson: my main supervisor, principal investigator, SCCR-director, group leader, medical doctor, anatomy professor, researcher, morphologist, microscopist, progenitor (*sic!*), inspirer, deliverer, frustrator, brutal truth-teller, yes-sayer, BMW-driver, cat-lover, writing-long-sentences-master, often-booking-the-delayed-flight-traveller, always keeping the door open, never dismissing my whims (well, almost), never discouraging, always a teacher, loyal colleague – and friend. Thank you!

Therese Carlsson: my arms and legs, the glue, the security, the experience, the knowledge, the patience, the talks, the laughs, the mood swings, the families, the podcasts, the conferences, the hangovers (for you, I'm just sleep deprived!), the weight watchers, the supply orderings, the sectionings, the stainings, the experiments, the stupid questions (from me!), the lunches, the morning coffees, the afternoon cravings – the days of our lives, I wouldn't change them for anything.

Henrik Fagman: co-supervisor and colleague, whose opinions I value highly. I think you personify the complexity of crosstalk between germ layers!

Martin O Bergö: my other co-supervisor, thank you for valuable inputs and profound knowledge! Why do we always seem to meet in the elevator?

Karin Larsson: the sun in the gloomy mornings (which you know I hate)! If everyone had your spirit and motivation, I think we could cure cancer and accomplish world peace within the same year. Now, retire and enjoy!

Ellen Johansson: my precursor and recruiter, paving the way and being an ally on so many levels within research, medicine, teaching, and academia. The order in my chaos, the blue to my red. Everything will be fine once it's painted.

Shawn Liang: my other precursor, teaching me all there is to know about breeding (mice!). Thank you for all the help and expertise! I think I've stolen your deskspace….

Jakob Dahlberg: thanks for letting me hold a Langenbeck from time to time! When are you coming to the animal house with me?

Carmen Moccia: you vibrant, Italian Gaia! Gothenburg was too small for you!

Junchi Huang and Sandra Ferreyra Vega: I would never have made it through without you! Thanks for all the laughs, late-night experiments, antibodies, Fisherman's, hugs, Excel rescues…

Mikael Montelius: who would have guessed that a Christmas lunch would render me WANTING to understand the physics of magnetic resonance!

Bengt R Johansson: my dear mentor and anatomy professor, expert in anatomy, electron microscopy, barbecuing and verbal bullying ;).

Anne Uv and **Magnus Braide**: true academics, loyal colleagues, and great friends!

Emma, Claudia and **Jonna** who all have made sure that our mice are taken care of properly - thank you!

At this point you probably all know that my brain is fried, and I don't know who to mention, and I will probably forget half of you, so I will just say that I am so happy to have you all in my life and I look forward to future collaborations, after works, late nights in the lab, lunches and so on. No more joint pregnancies though **Anna Linder**.

I especially want to thank **Göteborgs Läkaresällskap** and **Assar Gabrielssons fond** for funding me generously throughout the years.

Finally, I want to thank my family, especially **Anton** and **Linnea** - no more boring mother sitting by the computer day and night! Not for a while at least… **Margareta Andersson**: my BFF, personal trainer, personal shopper, lifesaver. And **Christian**: the reason for me being here at all.

REFERENCES

1. Solnica-Krezel, L. and D.S. Sepich, *Gastrulation: making and shaping germ layers.* Annu Rev Cell Dev Biol, 2012. **28**: p. 687-717.
2. Pijuan-Sala, B., et al., *A single-cell molecular map of mouse gastrulation and early organogenesis.* Nature, 2019. **566**(7745): p. 490-495.
3. Han, L., et al., *Single cell transcriptomics identifies a signaling network coordinating endoderm and mesoderm diversification during foregut organogenesis.* Nat Commun, 2020. **11**(1): p. 4158.
4. Fagman, H., et al., *Gene expression profiling at early organogenesis reveals both common and diverse mechanisms in foregut patterning.* Dev Biol, 2011. **359**(2): p. 163-75.
5. Liao, B.Y. and J. Zhang, *Low rates of expression profile divergence in highly expressed genes and tissue-specific genes during mammalian evolution.* Mol Biol Evol, 2006. **23**(6): p. 1119-28.
6. Nilsson, M. and H. Fagman, *Development of the thyroid gland.* Development, 2017. **144**(12): p. 2123-2140.
7. Trueba, S.S., et al., *PAX8, TITF1, and FOXE1 gene expression patterns during human development: new insights into human thyroid development and thyroid dysgenesis-associated malformations.* J Clin Endocrinol Metab, 2005. **90**(1): p. 455-62.
8. Zorn, A.M. and J.M. Wells, *Vertebrate endoderm development and organ formation.* Annu Rev Cell Dev Biol, 2009. **25**: p. 221-51.
9. Frisdal, A. and P.A. Trainor, *Development and evolution of the pharyngeal apparatus.* Wiley Interdiscip Rev Dev Biol, 2014. **3**(6): p. 403-18.
10. Brent, G.A., *The molecular basis of thyroid hormone action.* N Engl J Med, 1994. **331**(13): p. 847-53.
11. Chiamolera, M.I. and F.E. Wondisford, *Minireview: Thyrotropin-releasing hormone and the thyroid hormone feedback mechanism.* Endocrinology, 2009. **150**(3): p. 1091-6.
12. Palmer, W.W., *Metabolism in Hyperthyroidism and Hypothyroidism.* Bull N Y Acad Med, 1934. **10**(2): p. 52-64.
13. Ericson, L.E. and M. Nilsson, *Structural and functional aspects of the thyroid follicular epithelium.* Toxicol Lett, 1992. **64-65 Spec No**: p. 365-73.
14. Nilsson, M., H. Fagman, and L.E. Ericson, *Ca2+-dependent and Ca2+-independent regulation of the thyroid epithelial junction complex by protein kinases.* Exp Cell Res, 1996. **225**(1): p. 1-11.
15. Citterio, C.E., H.M. Targovnik, and P. Arvan, *The role of thyroglobulin in thyroid hormonogenesis.* Nat Rev Endocrinol, 2019. **15**(6): p. 323-338.
16. Dai, G., O. Levy, and N. Carrasco, *Cloning and characterization of the thyroid iodide transporter.* Nature, 1996. **379**(6564): p. 458-60.

17. Carvalho, D.P. and C. Dupuy, *Thyroid hormone biosynthesis and release.* Mol Cell Endocrinol, 2017. **458**: p. 6-15.
18. Dumont, J.E., et al., *Physiological and pathological regulation of thyroid cell proliferation and differentiation by thyrotropin and other factors.* Physiol Rev, 1992. **72**(3): p. 667-97.
19. Postiglione, M.P., et al., *Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15462-7.
20. Schaller, R.T., Jr. and J.K. Stevenson, *Development of carcinoma of the thyroid in iodine-deficient mice.* Cancer, 1966. **19**(8): p. 1063-80.
21. Franco, A.T., et al., *Thyrotrophin receptor signaling dependence of Braf-induced thyroid tumor initiation in mice.* Proc Natl Acad Sci U S A, 2011. **108**(4): p. 1615-20.
22. Charles, R.P., et al., *Mutationally activated BRAF(V600E) elicits papillary thyroid cancer in the adult mouse.* Cancer Res, 2011. **71**(11): p. 3863-71.
23. Knauf, J.A., et al., *Targeted expression of BRAFV600E in thyroid cells of transgenic mice results in papillary thyroid cancers that undergo dedifferentiation.* Cancer Res, 2005. **65**(10): p. 4238-45.
24. Studer, H., H.J. Peter, and H. Gerber, *Natural heterogeneity of thyroid cells: the basis for understanding thyroid function and nodular goiter growth.* Endocr Rev, 1989. **10**(2): p. 125-35.
25. Johansson, E., et al., *Asynchrony of Apical Polarization, Luminogenesis, and Functional Differentiation in the Developing Thyroid Gland.* Front Endocrinol (Lausanne), 2021. **12**: p. 760541.
26. Fagman, H., L. Andersson, and M. Nilsson, *The developing mouse thyroid: embryonic vessel contacts and parenchymal growth pattern during specification, budding, migration, and lobulation.* Dev Dyn, 2006. **235**(2): p. 444-55.
27. Fernández, L.P., A. López-Márquez, and P. Santisteban, *Thyroid transcription factors in development, differentiation and disease.* Nat Rev Endocrinol, 2015. **11**(1): p. 29-42.
28. Johansson, E., et al., *Revising the embryonic origin of thyroid C cells in mice and humans.* Development, 2015. **142**(20): p. 3519-28.
29. Liang, S., et al., *A branching morphogenesis program governs embryonic growth of the thyroid gland.* Development, 2018. **145**(2).
30. Metzger, R.J., et al., *The branching programme of mouse lung development.* Nature, 2008. **453**(7196): p. 745-50.
31. Pan, H., G.H. Deutsch, and S.E. Wert, *Comprehensive anatomic ontologies for lung development: A comparison of alveolar formation and maturation within mouse and human lung.* J Biomed Semantics, 2019. **10**(1): p. 18.
32. Vandam, L.D., *The functional anatomy of the lung.* Anesthesiology, 1952. **13**(2): p. 130-41.

33. Huber, J.F., *Practical correlative anatomy of the bronchial tree and lungs.* J Natl Med Assoc, 1949. **41**(2): p. 49-60.
34. De Groodt, M., A. Lagasse, and M. Sebruyns, *Fine structure of the alveolar wall of the lung.* Nature, 1958. **181**(4615): p. 1066-7.
35. Travaglini, K.J., et al., *A molecular cell atlas of the human lung from single-cell RNA sequencing.* Nature, 2020. **587**(7835): p. 619-625.
36. Franks, T.J., et al., *Resident cellular components of the human lung: current knowledge and goals for research on cell phenotyping and function.* Proc Am Thorac Soc, 2008. **5**(7): p. 763-6.
37. Schittny, J.C., *Development of the lung.* Cell Tissue Res, 2017. **367**(3): p. 427-444.
38. Bellusci, S., et al., *Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung.* Development, 1997. **124**(23): p. 4867-78.
39. Min, H., et al., *Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless.* Genes Dev, 1998. **12**(20): p. 3156-61.
40. Fagman, H., et al., *Genetic deletion of sonic hedgehog causes hemiagenesis and ectopic development of the thyroid in mouse.* Am J Pathol, 2004. **164**(5): p. 1865-72.
41. Cardoso, W.V. and J.A. Whitsett, *Resident cellular components of the lung: developmental aspects.* Proc Am Thorac Soc, 2008. **5**(7): p. 767-71.
42. López-Márquez, A., et al., *Unraveling the Complex Interplay Between Transcription Factors and Signaling Molecules in Thyroid Differentiation and Function, From Embryos to Adults.* Front Endocrinol (Lausanne), 2021. **12**: p. 654569.
43. Krude, H., et al., *Choreoathetosis, hypothyroidism, and pulmonary alterations due to human NKX2-1 haploinsufficiency.* J Clin Invest, 2002. **109**(4): p. 475-80.
44. Patel, N.J. and J. Jankovic, *NKX2-1-Related Disorders*, in *GeneReviews(®)*, M.P. Adam, et al., Editors. 1993, University of Washington, Seattle
45. Kimura, S., et al., *The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary.* Genes Dev, 1996. **10**(1): p. 60-9.
46. Kusakabe, T., N. Hoshi, and S. Kimura, *Origin of the ultimobranchial body cyst: T/ebp/Nkx2.1 expression is required for development and fusion of the ultimobranchial body to the thyroid.* Dev Dyn, 2006. **235**(5): p. 1300-9.
47. Kusakabe, T., et al., *Thyroid-specific enhancer-binding protein/NKX2.1 is required for the maintenance of ordered architecture and function of the differentiated thyroid.* Mol Endocrinol, 2006. **20**(8): p. 1796-809.

48. DeFelice, M., et al., *TTF-1 phosphorylation is required for peripheral lung morphogenesis, perinatal survival, and tissue-specific gene expression.* J Biol Chem, 2003. **278**(37): p. 35574-83.
49. Silberschmidt, D., et al., *In vivo role of different domains and of phosphorylation in the transcription factor Nkx2-1.* BMC Dev Biol, 2011. **11**: p. 9.
50. Lazzaro, D., et al., *The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain.* Development, 1991. **113**(4): p. 1093-104.
51. Stahlman, M.T., M.E. Gray, and J.A. Whitsett, *Expression of thyroid transcription factor-1(TTF-1) in fetal and neonatal human lung.* J Histochem Cytochem, 1996. **44**(7): p. 673-8.
52. Boggaram, V., *Regulation of lung surfactant protein gene expression.* Front Biosci, 2003. **8**: p. d751-64.
53. Zhou, J., et al., *Non-medullary Thyroid Cancer Susceptibility Genes: Evidence and Disease Spectrum.* Ann Surg Oncol, 2021. **28**(11): p. 6590-6600.
54. Nonaka, D., et al., *Diagnostic utility of thyroid transcription factors Pax8 and TTF-2 (FoxE1) in thyroid epithelial neoplasms.* Mod Pathol, 2008. **21**(2): p. 192-200.
55. Travis, W.D., et al., *The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances Since the 2004 Classification.* J Thorac Oncol, 2015. **10**(9): p. 1243-1260.
56. Tan, D., et al., *Thyroid transcription factor-1 expression prevalence and its clinical implications in non-small cell lung cancer: a high-throughput tissue microarray and immunohistochemistry study.* Hum Pathol, 2003. **34**(6): p. 597-604.
57. Puglisi, F., et al., *Prognostic value of thyroid transcription factor-1 in primary, resected, non-small cell lung carcinoma.* Mod Pathol, 1999. **12**(3): p. 318-24.
58. Kwei, K.A., et al., *Genomic profiling identifies TITF1 as a lineage-specific oncogene amplified in lung cancer.* Oncogene, 2008. **27**(25): p. 3635-40.
59. Yamaguchi, T., et al., *NKX2-1/TTF-1: an enigmatic oncogene that functions as a double-edged sword for cancer cell survival and progression.* Cancer Cell, 2013. **23**(6): p. 718-23.
60. Mu, D., *The complexity of thyroid transcription factor 1 with both pro- and anti-oncogenic activities.* J Biol Chem, 2013. **288**(35): p. 24992-25000.
61. Zhang P.et al.,*Immunohistochemical analysis of thyroid-specific transcription factors in thyroid tumors.* Pathol Int, 2006. **56**(5): p.240-5.
62. Ros, P., et al., *Thyroid-specific gene expression in the multi-step process of thyroid carcinogenesis.* Biochimie, 1999. **81**(4): p. 389-96.

63. Fugazzola, L., et al., *2019 European Thyroid Association Guidelines for the Treatment and Follow-Up of Advanced Radioiodine-Refractory Thyroid Cancer.* Eur Thyroid J, 2019. **8**(5): p. 227-245.
64. Furuya, F., et al., *Adenovirus-mediated transfer of thyroid transcription factor-1 induces radioiodide organification and retention in thyroid cancer cells.* Endocrinology, 2004. **145**(11): p. 5397-405.
65. Mansouri, A., M. Hallonet, and P. Gruss, *Pax genes and their roles in cell differentiation and development.* Curr Opin Cell Biol, 1996. **8**(6): p. 851-7.
66. Mansouri, A., L. St-Onge, and P. Gruss, *Role of Genes in Endoderm-derived Organs.* Trends Endocrinol Metab, 1999. **10**(4): p. 164-167.
67. Plachov, D., et al., *Pax8, a murine paired box gene expressed in the developing excretory system and thyroid gland.* Development, 1990. **110**(2): p. 643-51.
68. Vainio, S., et al., *Female development in mammals is regulated by Wnt-4 signalling.* Nature, 1999. **397**(6718): p. 405-9.
69. Mansouri, A., K. Chowdhury, and P. Gruss, *Follicular cells of the thyroid gland require Pax8 gene function.* Nat Genet, 1998. **19**(1): p. 87-90.
70. Parlato, R., et al., *An integrated regulatory network controlling survival and migration in thyroid organogenesis.* Dev Biol, 2004. **276**(2): p. 464-75.
71. De Felice, M. and R. Di Lauro, *Thyroid development and its disorders: genetics and molecular mechanisms.* Endocr Rev, 2004. **25**(5): p. 722-46.
72. Mittag, J., et al., *Congenital hypothyroid female pax8-deficient mice are infertile despite thyroid hormone replacement therapy.* Endocrinology, 2007. **148**(2): p. 719-25.
73. Marotta, P., et al., *The paired box transcription factor Pax8 is essential for function and survival of adult thyroid cells.* Mol Cell Endocrinol, 2014. **396**(1-2): p. 26-36.
74. Laury, A.R., et al., *A comprehensive analysis of PAX8 expression in human epithelial tumors.* Am J Surg Pathol, 2011. **35**(6): p. 816-26.
75. Vidarsdottir, H., et al., *Immunohistochemical profiles in primary lung cancers and epithelial pulmonary metastases.* Hum Pathol, 2019. **84**: p. 221-230.
76. Zannini, M., et al., *TTF-2, a new forkhead protein, shows a temporal expression in the developing thyroid which is consistent with a role in controlling the onset of differentiation.* Embo j, 1997. **16**(11): p. 3185-97.
77. De Felice, M., et al., *A mouse model for hereditary thyroid dysgenesis and cleft palate.* Nat Genet, 1998. **19**(4): p. 395-8.
78. Zhu, H., et al., *Quantitative assessment of common genetic variants on FOXE1 and differentiated thyroid cancer risk.* PLoS One, 2014. **9**(1): p. e87332.
79. Credendino, S.C., et al., *FOXE1 Gene Dosage Affects Thyroid Cancer Histology and Differentiation In Vivo.* Int J Mol Sci, 2020. **22**(1).

80. Ji, G.H., et al., *Profiling analysis of FOX gene family members identified FOXE1 as potential regulator of NSCLC development.* Cell Mol Biol (Noisy-le-grand), 2016. **62**(11): p. 57-62.
81. Bogue, C.W., et al., *Hex expression suggests a role in the development and function of organs derived from foregut endoderm.* Dev Dyn, 2000. **219**(1): p. 84-9.
82. Martinez Barbera, J.P., et al., *The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation.* Development, 2000. **127**(11): p. 2433-45.
83. Golson, M.L. and K.H. Kaestner, *Fox transcription factors: from development to disease.* Development, 2016. **143**(24): p. 4558-4570.
84. Wan, H., et al., *Compensatory roles of Foxa1 and Foxa2 during lung morphogenesis.* J Biol Chem, 2005. **280**(14): p. 13809-16.
85. Waterston, R.H., et al., *Initial sequencing and comparative analysis of the mouse genome.* Nature, 2002. **420**(6915): p. 520-62.
86. Einarsdottir, B.O., et al., *Melanoma patient-derived xenografts accurately model the disease and develop fast enough to guide treatment decisions.* Oncotarget, 2014. **5**(20): p. 9609-18.
87. Talmadge, J.E., et al., *Murine models to evaluate novel and conventional therapeutic strategies for cancer.* Am J Pathol, 2007. **170**(3): p. 793-804.
88. Alfred, L.J., et al., *A chemical carcinogen, 3-methylcholanthrene, alters T-cell function and induces T-suppressor cells in a mouse model system.* Immunology, 1983. **50**(2): p. 207-13.
89. Rivina, L. and R. Schiestl, *Mouse models of radiation-induced cancers.* Adv Genet, 2013. **84**: p. 83-122.
90. Thomas, K.R. and M.R. Capecchi, *Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells.* Cell, 1987. **51**(3): p. 503-12.
91. Donehower, L.A., et al., *Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours.* Nature, 1992. **356**(6366): p. 215-21.
92. Brinster, R.L., et al., *Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors.* Cell, 1984. **37**(2): p. 367-79.
93. Agarwal, S.K., *Exploring the tumors of multiple endocrine neoplasia type 1 in mouse models for basic and preclinical studies.* Int J Endocr Oncol, 2014. **1**(2): p. 153-161.
94. Ledermann, B., *Embryonic stem cells and gene targeting.* Exp Physiol, 2000. **85**(6): p. 603-13.
95. Cabanillas, M.E., D.G. McFadden, and C. Durante, *Thyroid cancer.* Lancet, 2016. **388**(10061): p. 2783-2795.
96. Graham, A., *MALIGNANT TUMORS OF THE THYROID: EPITHELIAL TYPES.* Ann Surg, 1925. **82**(1): p. 30-44.
97. Wilson, L.B., *MALIGNANT TUMORS OF THE THYROID.* Ann Surg, 1921. **74**(2): p. 129-84.

98. Dralle, H., et al., *Follicular cell-derived thyroid cancer.* Nat Rev Dis Primers, 2015. **1**: p. 15077.
99. Prete, A., et al., *Update on Fundamental Mechanisms of Thyroid Cancer.* Front Endocrinol (Lausanne), 2020. **11**: p. 102.
100. Volante, M. and M. Papotti, *Poorly differentiated thyroid carcinoma: 5 years after the 2004 WHO classification of endocrine tumours.* Endocr Pathol, 2010. **21**(1): p. 1-6.
101. Volante, M., et al., *Poorly differentiated thyroid carcinoma: the Turin proposal for the use of uniform diagnostic criteria and an algorithmic diagnostic approach.* Am J Surg Pathol, 2007. **31**(8): p. 1256-64.
102. Molinaro, E., et al., *Anaplastic thyroid carcinoma: from clinicopathology to genetics and advanced therapies.* Nat Rev Endocrinol, 2017. **13**(11): p. 644-660.
103. Leboulleux, S., et al., *Medullary thyroid carcinoma.* Clin Endocrinol (Oxf), 2004. **61**(3): p. 299-310.
104. Kitahara, C.M. and J.A. Sosa, *The changing incidence of thyroid cancer.* Nat Rev Endocrinol, 2016. **12**(11): p. 646-653.
105. La Vecchia, C., et al., *Thyroid cancer mortality and incidence: a global overview.* Int J Cancer, 2015. **136**(9): p. 2187-95.
106. Vaccarella, S., et al., *Worldwide Thyroid-Cancer Epidemic? The Increasing Impact of Overdiagnosis.* N Engl J Med, 2016. **375**(7): p. 614-7.
107. Dahlberg, J., et al., *Incidence, detection and outcome of differentiated thyroid cancer in Western Sweden.* BJS Open, 2021. **5**(5).
108. La Vecchia, C. and E. Negri, *Thyroid cancer: The thyroid cancer epidemic - overdiagnosis or a real increase?* Nat Rev Endocrinol, 2017. **13**(6): p. 318-319.
109. Lim, H., et al., *Trends in Thyroid Cancer Incidence and Mortality in the United States, 1974-2013.* Jama, 2017. **317**(13): p. 1338-1348.
110. Nikiforov, Y.E., *Radiation-induced thyroid cancer: what we have learned from chernobyl.* Endocr Pathol, 2006. **17**(4): p. 307-17.
111. Williams, D., *Cancer after nuclear fallout: lessons from the Chernobyl accident.* Nat Rev Cancer, 2002. **2**(7): p. 543-9.
112. Kitahara, C.M., et al., *Impact of Overweight and Obesity on US Papillary Thyroid Cancer Incidence Trends (1995-2015).* J Natl Cancer Inst, 2020. **112**(8): p. 810-817.
113. Rahman, S.T., et al., *Obesity Is Associated with BRAF(V600E)-Mutated Thyroid Cancer.* Thyroid, 2020. **30**(10): p. 1518-1527.
114. Haugen, B.R., et al., *2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer.* Thyroid, 2016. **26**(1): p. 1-133.

115. Russ, G., et al., *European Thyroid Association Guidelines for Ultrasound Malignancy Risk Stratification of Thyroid Nodules in Adults: The EU-TIRADS.* Eur Thyroid J, 2017. **6**(5): p. 225-237.
116. Cibas, E.S. and S.Z. Ali, *The Bethesda System For Reporting Thyroid Cytopathology.* Am J Clin Pathol, 2009. **132**(5): p. 658-65.
117. Maletta, F., et al., *Cytological features of "noninvasive follicular thyroid neoplasm with papillary-like nuclear features" and their correlation with tumor histology.* Hum Pathol, 2016. **54**: p. 134-42.
118. Nikiforova, M.N., et al., *Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules.* Cancer, 2018. **124**(8): p. 1682-1690.
119. Kim, H., et al., *Pattern analysis for prognosis of differentiated thyroid cancer according to preoperative serum thyrotropin levels.* Sci Rep, 2021. **11**(1): p. 22322.
120. Haymart, M.R., et al., *Higher serum thyroid stimulating hormone level in thyroid nodule patients is associated with greater risks of differentiated thyroid cancer and advanced tumor stage.* J Clin Endocrinol Metab, 2008. **93**(3): p. 809-14.
121. Polyzos, S.A., et al., *Serum thyrotropin concentration as a biochemical predictor of thyroid malignancy in patients presenting with thyroid nodules.* J Cancer Res Clin Oncol, 2008. **134**(9): p. 953-60.
122. King, A.D., *Imaging for staging and management of thyroid cancer.* Cancer Imaging, 2008. **8**(1): p. 57-69.
123. Eustatia-Rutten, C.F., et al., *Diagnostic value of serum thyroglobulin measurements in the follow-up of differentiated thyroid carcinoma, a structured meta-analysis.* Clin Endocrinol (Oxf), 2004. **61**(1): p. 61-74.
124. Dhillon, A.S., et al., *MAP kinase signalling pathways in cancer.* Oncogene, 2007. **26**(22): p. 3279-90.
125. Zaballos, M.A. and P. Santisteban, *Key signaling pathways in thyroid cancer.* J Endocrinol, 2017. **235**(2): p. R43-r61.
126. Nikiforov, Y.E. and M.N. Nikiforova, *Molecular genetics and diagnosis of thyroid cancer.* Nat Rev Endocrinol, 2011. **7**(10): p. 569-80.
127. Robinson, M.J. and M.H. Cobb, *Mitogen-activated protein kinase pathways.* Curr Opin Cell Biol, 1997. **9**(2): p. 180-6.
128. Brummer, T., et al., *Functional analysis of the regulatory requirements of B-Raf and the B-Raf(V600E) oncoprotein.* Oncogene, 2006. **25**(47): p. 6262-76.
129. Baloch, Z.W., et al., *Overview of the 2022 WHO Classification of Thyroid Neoplasms.* Endocr Pathol, 2022. **33**(1): p. 27-63.
130. Xing, M., *BRAF mutation in thyroid cancer.* Endocr Relat Cancer, 2005. **12**(2): p. 245-62.
131. Xing, M., *BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications.* Endocr Rev, 2007. **28**(7): p. 742-62.

132. Cohen, Y., et al., *BRAF mutation in papillary thyroid carcinoma.* J Natl Cancer Inst, 2003. **95**(8): p. 625-7.
133. Kimura, E.T., et al., *High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma.* Cancer Res, 2003. **63**(7): p. 1454-7.
134. Cui, G., et al., *A meta-analysis of the association between BRAF mutation and nonsmall cell lung cancer.* Medicine (Baltimore), 2017. **96**(14): p. e6552.
135. Hall, R.D. and R.R. Kudchadkar, *BRAF mutations: signaling, epidemiology, and clinical experience in multiple malignancies.* Cancer Control, 2014. **21**(3): p. 221-30.
136. Barras, D., *BRAF Mutation in Colorectal Cancer: An Update.* Biomark Cancer, 2015. **7**(Suppl 1): p. 9-12.
137. Santarpia, L., et al., *Genetic alterations in the RAS/RAF/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways in the follicular variant of papillary thyroid carcinoma.* Cancer, 2010. **116**(12): p. 2974-83.
138. Howell, G.M., S.P. Hodak, and L. Yip, *RAS mutations in thyroid cancer.* Oncologist, 2013. **18**(8): p. 926-32.
139. Rodenhuis, S., et al., *Mutational activation of the K-ras oncogene. A possible pathogenetic factor in adenocarcinoma of the lung.* N Engl J Med, 1987. **317**(15): p. 929-35.
140. Ciampi, R. and Y.E. Nikiforov, *RET/PTC rearrangements and BRAF mutations in thyroid tumorigenesis.* Endocrinology, 2007. **148**(3): p. 936-41.
141. Xing, M., *Molecular pathogenesis and mechanisms of thyroid cancer.* Nat Rev Cancer, 2013. **13**(3): p. 184-99.
142. Lloyd, R.V., D. Buehler, and E. Khanafshar, *Papillary thyroid carcinoma variants.* Head Neck Pathol, 2011. **5**(1): p. 51-6.
143. Omur, O. and Y. Baran, *An update on molecular biology of thyroid cancers.* Crit Rev Oncol Hematol, 2014. **90**(3): p. 233-52.
144. Zou, M., et al., *TSH overcomes Braf-induced senescence to promote tumor progression via downregulation of p53 expression in papillary thyroid cancer.* Oncogene, 2015.
145. Kitahara, C.M. and J.A. Sosa, *The changing incidence of thyroid cancer.* Nat Rev Endocrinol, 2016.
146. Li, P., et al., *Sex disparities in thyroid cancer: a SEER population study.* Gland Surg, 2021. **10**(12): p. 3200-3210.
147. *Integrated genomic characterization of papillary thyroid carcinoma.* Cell, 2014. **159**(3): p. 676-90.
148. Gray, A. and I. Doniach, *Morphology of the nuclei of papillary carcinoma of the thyroid.* Br J Cancer, 1969. **23**(1): p. 49-51.
149. Lindsay, S., *Natural history of thyroid carcinoma.* Ariz Med, 1960. **17**: p. 623-7.

150. Asioli, S., et al., *Papillary thyroid carcinoma with prominent hobnail features: a new aggressive variant of moderately differentiated papillary carcinoma. A clinicopathologic, immunohistochemical, and molecular study of eight cases.* Am J Surg Pathol, 2010. **34**(1): p. 44-52.
151. Davies, H., et al., *Mutations of the BRAF gene in human cancer.* Nature, 2002. **417**(6892): p. 949-54.
152. Orim, F., et al., *Thyrotropin signaling confers more aggressive features with higher genomic instability on BRAF(V600E)-induced thyroid tumors in a mouse model.* Thyroid, 2014. **24**(3): p. 502-10.
153. Xing, M., et al., *Association between BRAF V600E mutation and recurrence of papillary thyroid cancer.* J Clin Oncol, 2015. **33**(1): p. 42-50.
154. Kunstman, J.W., et al., *Characterization of the mutational landscape of anaplastic thyroid cancer via whole-exome sequencing.* Hum Mol Genet, 2015. **24**(8): p. 2318-29.
155. Russo, M.A., V.G. Antico Arciuch, and A. Di Cristofano, *Mouse models of follicular and papillary thyroid cancer progression.* Front Endocrinol (Lausanne), 2011. **2**: p. 119.
156. Vanden Borre, P., et al., *The next generation of orthotopic thyroid cancer models: immunocompetent orthotopic mouse models of BRAF V600E-positive papillary and anaplastic thyroid carcinoma.* Thyroid, 2014. **24**(4): p. 705-14.
157. McFadden, D.G., et al., *p53 constrains progression to anaplastic thyroid carcinoma in a Braf-mutant mouse model of papillary thyroid cancer.* Proc Natl Acad Sci U S A, 2014. **111**(16): p. E1600-9.
158. Moyzis, R.K., et al., *A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes.* Proc Natl Acad Sci U S A, 1988. **85**(18): p. 6622-6.
159. Blasco, M.A., *Telomeres and human disease: ageing, cancer and beyond.* Nat Rev Genet, 2005. **6**(8): p. 611-22.
160. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
161. Liu, R. and M. Xing, *TERT promoter mutations in thyroid cancer.* Endocr Relat Cancer, 2016. **23**(3): p. R143-55.
162. Liu, T., et al., *The age- and shorter telomere-dependent TERT promoter mutation in follicular thyroid cell-derived carcinomas.* Oncogene, 2014. **33**(42): p. 4978-84.
163. Juhlin, C.C., *A Clinical Overview of Telomerase-Associated Aberrancies in Follicular Thyroid Tumors as Diagnostic and Prognostic Markers: Tert Alert!* Scand J Surg, 2020. **109**(3): p. 187-192.
164. Filetti, S., et al., *Thyroid cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†.* Ann Oncol, 2019. **30**(12): p. 1856-1883.

165. Viola, D., et al., *Treatment of advanced thyroid cancer with targeted therapies: ten years of experience.* Endocr Relat Cancer, 2016. **23**(4): p. R185-205.
166. Hertz, S. and A. Roberts, *RADIOACTIVE IODINE AS AN INDICATOR IN THYROID PHYSIOLOGY. V. THE USE OF RADIOACTIVE IODINE IN THE DIFFERENTIAL DIAGNOSIS OF TWO TYPES OF GRAVES' DISEASE.* J Clin Invest, 1942. **21**(1): p. 31-2.
167. De La Vieja, A., et al., *Molecular analysis of the sodium/iodide symporter: impact on thyroid and extrathyroid pathophysiology.* Physiol Rev, 2000. **80**(3): p. 1083-105.
168. Dohan, O., et al., *The sodium/iodide Symporter (NIS): characterization, regulation, and medical significance.* Endocr Rev, 2003. **24**(1): p. 48-77.
169. Hertz, S. and A. Roberts, *Radioactive iodine in the study of thyroid physiology; the use of radioactive iodine therapy in hyperthyroidism.* J Am Med Assoc, 1946. **131**: p. 81-6.
170. Chapman, E.M. and R.D. Evans, *The treatment of hyperthyroidism with radioactive iodine.* J Am Med Assoc, 1946. **131**: p. 86-91.
171. Seidlin, S.M., L.D. Marinelli, and E. Oshry, *Radioactive iodine therapy; effect on functioning metastases of adenocarcinoma of the thyroid.* J Am Med Assoc, 1946. **132**(14): p. 838-47.
172. Sia, Y., et al., *Radioactive iodine ablation post differentiated thyroid cancer surgery: an analysis of use and impact of the American Thyroid Association guidelines.* ANZ J Surg, 2019. **89**(11): p. E502-e506.
173. Borges de Souza, P. and C.J. McCabe, *Radioiodine treatment: an historical and future perspective.* Endocr Relat Cancer, 2021. **28**(10): p. T121-t124.
174. Schlumberger, M., et al., *Defects in iodide metabolism in thyroid cancer and implications for the follow-up and treatment of patients.* Nat Clin Pract Endocrinol Metab, 2007. **3**(3): p. 260-9.
175. Romei, C., et al., *BRAFV600E mutation, but not RET/PTC rearrangements, is correlated with a lower expression of both thyroperoxidase and sodium iodide symporter genes in papillary thyroid cancer.* Endocr Relat Cancer, 2008. **15**(2): p. 511-20.
176. Lorusso, L., et al., *Thyroid Cancers: From Surgery to Current and Future Systemic Therapies through Their Molecular Identities.* Int J Mol Sci, 2021. **22**(6).
177. Cabanillas, M.E., M. Ryder, and C. Jimenez, *Targeted Therapy for Advanced Thyroid Cancer: Kinase Inhibitors and Beyond.* Endocr Rev, 2019. **40**(6): p. 1573-1604.
178. Subbiah, V., et al., *Dabrafenib and Trametinib Treatment in Patients With Locally Advanced or Metastatic BRAF V600-Mutant Anaplastic Thyroid Cancer.* J Clin Oncol, 2018. **36**(1): p. 7-13.
179. Tsai, J., et al., *Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 3041-6.

180. Kim, K.B., et al., *Clinical responses to vemurafenib in patients with metastatic papillary thyroid cancer harboring BRAF(V600E) mutation.* Thyroid, 2013. **23**(10): p. 1277-83.
181. Brose, M.S., et al., *Vemurafenib in patients with BRAF(V600E)-positive metastatic or unresectable papillary thyroid cancer refractory to radioactive iodine: a non-randomised, multicentre, open-label, phase 2 trial.* Lancet Oncol, 2016. **17**(9): p. 1272-82.
182. Dong, M., et al., *Sex Differences in Cancer Incidence and Survival: A Pan-Cancer Analysis.* Cancer Epidemiol Biomarkers Prev, 2020. **29**(7): p. 1389-1397.
183. Siegel, R.L., et al., *Cancer statistics, 2022.* CA Cancer J Clin, 2022. **72**(1): p. 7-33.
184. Madla, C.M., et al., *Let's talk about sex: Differences in drug therapy in males and females.* Adv Drug Deliv Rev, 2021. **175**: p. 113804.
185. Colonna, M., et al., *Progression of incidence and estimate of net survival from papillary thyroid cancers diagnosed between 2008 and 2016 in France.* Ann Endocrinol (Paris), 2020. **81**(6): p. 530-538.
186. LeClair, K., et al., *Evaluation of Gender Inequity in Thyroid Cancer Diagnosis: Differences by Sex in US Thyroid Cancer Incidence Compared With a Meta-analysis of Subclinical Thyroid Cancer Rates at Autopsy.* JAMA Intern Med, 2021. **181**(10): p. 1351-1358.
187. Farahati, J., et al., *Characteristics of differentiated thyroid carcinoma in children and adolescents with respect to age, gender, and histology.* Cancer, 1997. **80**(11): p. 2156-62.
188. Derwahl, M. and D. Nicula, *Estrogen and its role in thyroid cancer.* Endocr Relat Cancer, 2014. **21**(5): p. T273-83.
189. Molteni, A., et al., *Estradiol receptor-binding protein in head and neck neoplastic and normal tissue.* Arch Surg, 1981. **116**(2): p. 207-10.
190. Kuiper, G.G., et al., *Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta.* Endocrinology, 1997. **138**(3): p. 863-70.
191. Couse, J.F., et al., *Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse.* Endocrinology, 1997. **138**(11): p. 4613-21.
192. Santin, A.P. and T.W. Furlanetto, *Role of estrogen in thyroid function and growth regulation.* J Thyroid Res, 2011. **2011**: p. 875125.
193. Mauro, L.V., et al., *Estrogen receptor β and epidermal growth factor receptor as early-stage prognostic biomarkers of non-small cell lung cancer.* Oncol Rep, 2010. **24**(5): p. 1331-8.
194. Huang, Y., et al., *Differential expression patterns and clinical significance of estrogen receptor-α and β in papillary thyroid carcinoma.* BMC Cancer, 2014. **14**: p. 383.
195. Nicholson, A.G., et al., *The 2021 WHO Classification of Lung Tumors: Impact of Advances Since 2015.* J Thorac Oncol, 2022. **17**(3): p. 362-387.

196. Inamura, K., *Clinicopathological Characteristics and Mutations Driving Development of Early Lung Adenocarcinoma: Tumor Initiation and Progression.* Int J Mol Sci, 2018. **19**(4).
197. Hutchinson, B.D., et al., *Spectrum of Lung Adenocarcinoma.* Semin Ultrasound CT MR, 2019. **40**(3): p. 255-264.
198. Brose, M.S., et al., *BRAF and RAS mutations in human lung cancer and melanoma.* Cancer Res, 2002. **62**(23): p. 6997-7000.
199. da Cunha Santos, G., F.A. Shepherd, and M.S. Tsao, *EGFR mutations and lung cancer.* Annu Rev Pathol, 2011. **6**: p. 49-69.
200. Visvader, J.E., *Cells of origin in cancer.* Nature, 2011. **469**(7330): p. 314-22.
201. Rycaj, K. and D.G. Tang, *Cell-of-Origin of Cancer versus Cancer Stem Cells: Assays and Interpretations.* Cancer Res, 2015. **75**(19): p. 4003-11.
202. Giannini, R., et al., *The heterogeneous distribution of BRAF mutation supports the independent clonal origin of distinct tumor foci in multifocal papillary thyroid carcinoma.* J Clin Endocrinol Metab, 2007. **92**(9): p. 3511-6.
203. Fugazzola, L., et al., *Intratumoral Genetic Heterogeneity in Papillary Thyroid Cancer: Occurrence and Clinical Significance.* Cancers (Basel), 2020. **12**(2).
204. Turajlic, S., et al., *Resolving genetic heterogeneity in cancer.* Nat Rev Genet, 2019. **20**(7): p. 404-416.
205. Nowell, P.C., *The clonal evolution of tumor cell populations.* Science, 1976. **194**(4260): p. 23-8.
206. Swanton, C., *Intratumor heterogeneity: evolution through space and time.* Cancer Res, 2012. **72**(19): p. 4875-82.
207. Williams, D., *Thyroid Growth and Cancer.* Eur Thyroid J, 2015. **4**(3): p. 164-73.
208. Dankort, D., et al., *Braf(V600E) cooperates with Pten loss to induce metastatic melanoma.* Nat Genet, 2009. **41**(5): p. 544-52.
209. Rajagopalan, H., et al., *Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status.* Nature, 2002. **418**(6901): p. 934.
210. Medzhitov, R., *Origin and physiological roles of inflammation.* Nature, 2008. **454**(7203): p. 428-35.
211. Balkwill, F. and A. Mantovani, *Inflammation and cancer: back to Virchow?* Lancet, 2001. **357**(9255): p. 539-45.
212. Roma-Rodrigues, C., et al., *Targeting Tumor Microenvironment for Cancer Therapy.* Int J Mol Sci, 2019. **20**(4).
213. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.
214. Ferrari, S.M., et al., *Thyroid autoimmune disorders and cancer.* Semin Cancer Biol, 2020. **64**: p. 135-146.
215. Menicali, E., et al., *Immune Landscape of Thyroid Cancers: New Insights.* Front Endocrinol (Lausanne), 2020. **11**: p. 637826.

216. Russell, J.P., J.B. Engiles, and J.L. Rothstein, *Proinflammatory mediators and genetic background in oncogene mediated tumor progression.* J Immunol, 2004. **172**(7): p. 4059-67.
217. Sternberg, N. and D. Hamilton, *Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites.* J Mol Biol, 1981. **150**(4): p. 467-86.
218. Smedley, D., E. Salimova, and N. Rosenthal, *Cre recombinase resources for conditional mouse mutagenesis.* Methods, 2011. **53**(4): p. 411-6.
219. Dankort, D., et al., *A new mouse model to explore the initiation, progression, and therapy of BRAFV600E-induced lung tumors.* Genes Dev, 2007. **21**(4): p. 379-84.
220. Nagy, A., L. Mar, and G. Watts, *Creation and use of a cre recombinase transgenic database.* Methods Mol Biol, 2009. **530**: p. 365-78.
221. Wang, X., *Cre transgenic mouse lines.* Methods Mol Biol, 2009. **561**: p. 265-73.
222. Ng, P., et al., *A high-efficiency Cre/loxP-based system for construction of adenoviral vectors.* Hum Gene Ther, 1999. **10**(16): p. 2667-72.
223. Feil, R., et al., *Ligand-activated site-specific recombination in mice.* Proc Natl Acad Sci U S A, 1996. **93**(20): p. 10887-90.
224. Indra, A.K., et al., *Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases.* Nucleic Acids Res, 1999. **27**(22): p. 4324-7.
225. Xu, Q., M. Tam, and S.A. Anderson, *Fate mapping Nkx2.1-lineage cells in the mouse telencephalon.* J Comp Neurol, 2008. **506**(1): p. 16-29.
226. Undeutsch, H., et al., *A mouse model with tamoxifen-inducible thyrocyte-specific cre recombinase activity.* Genesis, 2014. **52**(4): p. 333-40.
227. Taniguchi, H., et al., *A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex.* Neuron, 2011. **71**(6): p. 995-1013.
228. Turlo, K.A., et al., *When Cre-mediated recombination in mice does not result in protein loss.* Genetics, 2010. **186**(3): p. 959-67.
229. Heffner, C.S., et al., *Supporting conditional mouse mutagenesis with a comprehensive cre characterization resource.* Nat Commun, 2012. **3**: p. 1218.
230. Kristianto, J., et al., *Spontaneous recombinase activity of Cre-ERT2 in vivo.* Transgenic Res, 2017. **26**(3): p. 411-417.
231. Muzumdar, M.D., et al., *A global double-fluorescent Cre reporter mouse.* Genesis, 2007. **45**(9): p. 593-605.
232. van Isselt, J.W., et al., *Comparison of methods for thyroid volume estimation in patients with Graves' disease.* Eur J Nucl Med Mol Imaging, 2003. **30**(4): p. 525-31.
233. Bozgeyik, Z., et al., *Diffusion-weighted MR imaging of thyroid nodules.* Neuroradiology, 2009. **51**(3): p. 193-8.

234. Montelius, M., et al., *Tumour size measurement in a mouse model using high resolution MRI.* BMC Med Imaging, 2012. **12**: p. 12.
235. Pouget, J.P., et al., *Introduction to radiobiology of targeted radionuclide therapy.* Front Med (Lausanne), 2015. **2**: p. 12.
236. Ståhlberg, A., et al., *Simple multiplexed PCR-based barcoding of DNA for ultrasensitive mutation detection by next-generation sequencing.* Nat Protoc, 2017. **12**(4): p. 664-682.
237. Kim, G., et al., *FDA approval summary: vemurafenib for treatment of unresectable or metastatic melanoma with the BRAFV600E mutation.* Clin Cancer Res, 2014. **20**(19): p. 4994-5000.
238. Oneal, P.A., et al., *FDA Approval Summary: Vemurafenib for the Treatment of Patients with Erdheim-Chester Disease with the BRAFV600 Mutation.* Oncologist, 2018. **23**(12): p. 1520-1524.
239. Liu, J., et al., *Non-parallel recombination limits Cre-LoxP-based reporters as precise indicators of conditional genetic manipulation.* Genesis, 2013. **51**(6): p. 436-42.
240. Park, S.Y. and J.S. Nam, *The force awakens: metastatic dormant cancer cells.* Exp Mol Med, 2020. **52**(4): p. 569-581.
241. Yuan, Y., et al., *Comprehensive Characterization of Molecular Differences in Cancer between Male and Female Patients.* Cancer Cell, 2016. **29**(5): p. 711-722.
242. Rubin, J.B., et al., *Sex differences in cancer mechanisms.* Biol Sex Differ, 2020. **11**(1): p. 17.

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