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REVERSAL OF THYROID DEDIFFERENTIATION AND AN INVASIVE PHENOTYPE BY SMALL MOLECULE KINASE INHIBITORS:

AN EXPERIMENTAL STUDY ON NORMAL AND MALIGNANT CELLS

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Till morfar Rurik

‘But I don’t want to go among mad people, ’ Alice remarked.
‘Oh, you can’t help that, ’ said the Cat: ‘we’re all mad here. I’m mad. You’re mad. ’
‘How do you know I’m mad?’ said Alice.
‘You must be, ’ said the Cat, ‘or you wouldn’t have come here.

Lewis Carrol – Alice’s Adventures in Wonderland

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Abstract

Refractoriness to I-131 in dedifferentiated thyroid cancer is a great concern that restricts radioiodine therapy. There is also a lack of knowledge in understanding the mechanisms leading to repressed sodium iodide symporter (NIS) expression and impaired iodide uptake in tumor cells. With this background, *paper I* investigated how NIS and iodide transport in normal thyrocytes were affected during dedifferentiation induced by epidermal growth factor (EGF). This was done on highly differentiated thyroid epithelial cells cultured in low (0.5%) or high (5%) content of fetal bovine serum either on filter in bicameral inserts or embedded in 3D collagen gel. EGF abolished TSH-stimulated transcription of *NIS* in both type of cultures. U0126, a MEK inhibitor, reversed this effect but only in serum-starved 2D cultures. Inhibition of MAPK signaling failed to recover NIS-mediated iodide uptake in the presence of serum and in 3D-cultured follicles irrespective of serum. In contrast, EGF-induced down-regulation of thyroglobulin, the thyroid prohormone, was blocked by MEK inhibition. These findings suggest an additional mechanism besides the classical MAPK signaling that negatively regulates NIS and confer resistance to small molecule kinase inhibitors targeting the MAPK pathway in dedifferentiated thyroid cells.

In tumor progression cancer cells lose the ancestral epithelial phenotype and become invasive. Many mechanisms cooperate in this process including joint signaling of the MAPK and PI3K/AKT pathways, suggesting combined targeted treatment with kinase inhibitors would more effectively counteract invasiveness. This possibility was addressed in *paper II* in which cell migration into extracellular matrix from EGF-stimulated follicles was monitored during treatment with inhibitors of MEK (U0126) and PI3K (LY294002). Indeed, dual inhibition was required to prevent both cell proliferation and migration in response to EGF. Notably, single inhibition of PI3K adversely increased EGF-induced migration and invasion, probably by promoting disintegration of the follicular epithelium. As LY294002 did not compromise cell survival in the presence of EGF these findings call for caution in use of PI3K inhibitors as monotherapy of tumors with a constitutively activated MAPK pathway.

Activating $BRAF^{V600E}$ mutation is a common driver in thyroid cancer. Acquired drug resistance involving rebound activation of MAPK signaling restricts the promising possibility to treat BRAF mutant tumors with kinase-selective inhibitors as PLX4720. Combined drug treatment to overcome this is suggested. In *paper III* inhibitor efficacy on tumor cell migration was investigated in $BRAF^{V600E}$ mutant cell lines derived from papillary (BCPAP) and anaplastic (SW1736) thyroid cancer. Besides conventional scratch wounding a double-layered collagen gel model was developed for analysis of directed tumor cell invasion during prolonged culture. Both PLX4720 and U0126 inhibited BCPAP cell migration and reduced tumor cell viability in 3D culture. 2D migration of SW1736 cells resisted even combined drug treatment, whereas embedded in collagen gel both drugs reduced the invading cell numbers. However, dual inhibition of $BRAF^{V600E}$ and MEK did not prevent invasion although rebound activation of MAPK was blocked. This suggests presence of highly invasive tumor cell subclones in anaplastic cancer that escape targeted drug therapy due to MAPK independence.

Keywords: *Thyroid, cancer, NIS, MAPK, PI3K,* $BRAF^{V600E}$*, migration, 3D culture*

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List of Publications

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

I. **Ingeson Carlsson C, Nilsson M**
Switching from MAPK-dependent to MAPK-independent repression of the sodium-iodide symporter in 2D and 3D cultured normal thyroid cells
Mol Cell Endocrinol. 2013 Dec 5;381(1-2):241-54

II. **Ingeson Carlsson C, Nilsson M**
Dual contribution of MAPK and PI3K in epidermal growth factor-induced destabilization of thyroid follicular integrity and invasion of cells into extracellular matrix
Manuscript (submitted)

III. **Ingeson Carlsson C, Nilsson M**
Differential effects of MAPK pathway inhibitors on migration and invasiveness of $BRAF^{V600E}$ mutant thyroid cancer cells in 2D and 3D culture
Manuscript

Paper I was reprinted with permission from Elsevier and Molecular and Cellular Endocrinology.

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Abbreviations

AC	adenylate cyclase
AJ	adherens junction
ATC	anaplastic thyroid cancer
cAMP	3'5' cyclic adenosine monophosphate
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DIT	3,5´-diiodotyrosine
DTC	differentiated thyroid cancer
Duox1	dual oxidase1
Duox2	dual oxidase 2
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
ERK1/2	extracellular signal-related kinase 1/2
FTC	follicular thyroid cancer
GAP	guanosine activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
Grb2	growth factor receptor binding protein 2
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
IGF-1	insulin growth factor 1
IGF-R	insuling growth factor receptor
IP_3	inositol triphosphate
MAPK	mitogen-activated protein kinase
MCT8	monocarboxylate transporter 8
MIT	3-iodotyrosine
MKK	MAPK kinase
MKKK	MAPK kinase kinase
MMI	methimazole
MTC	medullary thyroid cancer
NIS	sodium iodide symporter
Pax8	paired box gene 8
PD	Potential difference
PDK1	phosphoinositide-dependent kinase 1
PDTC	poorly differentiated thyroid cancer
PI3K	phosphoinositide-3 kinase
PKA	protein kinase A
PLC	phospholipase C
PPARγ	peroxisome proliferator activated receptor γ
PTC	papillary thyroid cancer
PTEN	phosphatase tensin homolog
RSK	ribosomal S6 kinase
RTK	receptor tyrosine kinase
SH2	Src homology 2
SH3	Src homology 3
SOS1	sevenless homologue 1
T_3	triiodothyronine
T_4	tetraiodothyronine

TER	transepithelial resistance
TG	thyroglobulin
TGFβ	transforming growth factor beta
TJ	tight junction
TPO	thyroperoxidase
TR	thyroid hormone receptor
TRH	thyrotropin-releasing hormone
TSH	thyroid stimulating hormone
TSHR	thyrotropin stimulation hormone receptor
TTF1	thyroid transcription factor 1
TTF2	thyroid transcription factor 2
TβR-I	TGF beta receptor type I
TβR-II	TGF beta receptor type II
ZO-1	zonula occludens 1

The Thyroid Gland

The endocrine thyroid gland is located in the anterior neck. It consists of two lobes, left and right, on either side of trachea and connected to each other by the isthmus. There are several cell types found in the gland with approximately 70% of the total cell count composed of thyroid epithelial cells or the thyrocytes. Other frequent cells are fibroblasts and the endothelial cells forming the many capillaries in this highly vascularized organ. There are also a small number of parafollicular C cells (Dumont et al., 1992). The principal function of the thyroid gland is to produce and release thyroid hormones. Thyroid hormones, tetraiodothyronine (T_4) and the biologically more active triiodithyronine (T_3), are important for developmental growth and metabolism. T_3 acts through binding to thyroid hormone receptors (TRs) present in the nucleus of target cells thereby regulating gene transcription. Most cells in vertebrates are sensitive to thyroid hormones, although the effects vary considerably in between for example neurons, heart muscle and liver (Boelaert and Franklyn, 2005). The hormones are synthesized by the thyrocytes, which are organized in spherical structures, the follicles. These units are comprised of a single layer of follicular cells surrounding a lumen in which a protein enriched fluid, the colloid, is stored. The major constituent of the colloid is thyroglobulin (TG), the prohormone of T_3 and T_4, which will be more extensively discussed in later sections. The C cells, which produce calcitonin, are not investigated in this thesis and will not be discussed in more detail.

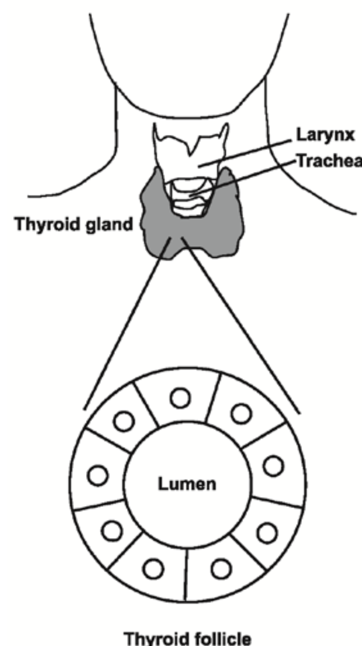

Fig. 1. Overview of the location and follicular organization of the thyroid gland in humans.

Thyrocytes differ from most endocrine cells in that they have features strongly reminiscent of secretory, exocrine cells. This includes a polarized plasma membrane that can be clearly divided into an apical and a basolateral domain with different constituent proteins residing there. The divergence in protein composition is maintained by intracellular sorting mechanisms (Rodriguez-Boulan and Nelson, 1989). Furthermore, the apical-basal polarity of epithelial cells is not only restricted to protein composition in the plasma membrane but is also manifested in the cells having a polarized distribution of organelles and also by way of specialized morphological features that can be found only on one side of the cell for example microvilli or pseudopods are present exclusively apically in thyroid cells. One additional feature of epithelial cells is the connections between the cells through different intercellular junctions. These includes tight junctions, adherens junctions and desmosomes originally described 50 years ago (Farquhar and Palade, 1963).

Epithelial Properties

The junctional features of the thyroid follicular epithelium are important regarding both structural organization of the follicle and the functional properties of individual thyroid cells. As both features were studied in papers I and II, it is relevant to describe some of these aspects in more detail.

Tight junctions

Tight junction (TJ) is the most apically localized junction and hence establishes a border between the basolateral and the apical parts of the cells (Farquhar and Palade, 1963). TJ can be said to have both a gate and a fence function. The gate feature arises from the importance of TJ in the regulation of paracellular passage of water, ion and molecules while the fence function is reflected by the role in maintaining the different protein and lipid composition in the apical and basolateral domains of the plasma membrane. Several proteins, both integral membrane and cytoplasmic, have been identified in the TJ complex. In the following sections the most prominent TJ proteins will be briefly overviewed. More comprehensive reviews of other TJ proteins for example tricellulin, PAR proteins, MUPP1, cingulin and symplekin etc., can be found in (Gonzalez-Mariscal et al., 2003; Gunzel and Fromm, 2012).

Occludin

The first TJ integral membrane protein discovered was occludin (Furuse et al., 1993). Occludin has four transmembrane regions, two extracellular loops and amino and carboxyl terminal end being found on the intracellular side. Despite the fact that its presence in TJ is apparent, the precise role is still unclear. This is especially emphasized by the fact that in occludin-deficient mice many epithelial organs and tissues still develop TJ (Saitou et al., 2000). Occludin can bind directly to F-actin through interaction with the carboxyl terminal end (Wittchen et al., 1999) a feature that differs from the other integral membrane TJ proteins that need adaptor proteins for the connection to the cytoskeleton. Occludin is expressed in thyrocytes (Grande et al., 2002).

Claudins

Another class of transmembrane proteins in the TJ is the claudin family first identified in 1998 (Furuse et al., 1998). Claudins are now considered to be the essential structural part in the TJ strands to which the other integral proteins on neighboring cells are associated in a homotypic fashion. So far, 27 mammalian claudin genes have been identified (Mineta et al., 2011) although there is a disagreement about whether the last three members reported should be classified as claudins (Maher et al., 2011). Claudins can interact through their extracellular loops with other claudins in the same membrane through *cis*-interactions and with claudins expressed by adjacent cells through *trans*-interactions. This leads to the formation of a zipper-like structure that contributes to the barrier (Piontek et al., 2008). Madin-Darby canine kidney (MDCK) commonly used in epithelial cell research consists of two strains with different expression patterns of claudins. The high-resistance type I cells express claudin-1 and claudin-4 while type II cells with leaky TJ also express claudin-2. Introduction of claudin-2 in type I cells caused the TJ to be leakier indicating that combinations and mixing ratios of different claudins give rise to variable tightness of the TJ (Furuse et al., 2001). The importance of claudins in the gate function of TJ is also demonstrated *in vivo* in claudin-1 deficient mice in which the affected epidermal barrier causes dehydration and early death due to excessive water loss (Furuse et al., 2002). Notably, thyroid epithelial cells, which establish a very tight epithelium reflecting the importance of keeping the follicle lumen secluded from the extra-follicular space, express claudin-1 (Grande et al., 2002).

ZO-1

ZO-1, named after the Latin word *zonula occludens* for TJ, was the first TJ protein to be identified in 1986 (Stevenson et al., 1986), and later on also ZO-2 (Jesaitis and Goodenough, 1994) and ZO-3 (Haskins et al., 1998) were identified. All three of them are cytoplasmatic proteins belonging to a protein family named membrane-associated guanylate kinase homologues or MAGUK. These proteins have structurally conserved PDZ, SH3 and GK domains. The PDZ domain is important for clustering and anchoring of transmembrane proteins (Kim et al., 1995) and proteins with several PDZ domains can function as a scaffold to bring different proteins together at a specific submembraneous location. ZO-1 has three PDZ domains, the first is associated with claudins (Itoh et al., 1999) and the other two bind to junctional adhesion molecules (JAM) representing yet another class of proteins found in TJ (Ebnet et al., 2000). In addition, ZO-1 is associated with occludin through the GK domain (Fanning et al., 1998; Schmidt et al., 2001) and to F-actin through its carboxyl-terminal end (Fanning et al., 1998; Itoh et al., 1997; Wittchen et al., 1999). As excellently reviewed by Tsukita (Tsukita et al., 2009), one of the leading scientists in this field, ZO-1 in joint action with ZO-2 serve as important organizers that are both required and sufficient for TJ formation and establishment of a paracellular barrier. In addition, ZO-1 interacts with cytoplasmatic proteins functioning in signal transduction and in regulation of gene expression by binding to the transcription factor ZO-1-associated nucleic acid-binding protein or ZONAB (Balda and Matter, 2000). Interestingly, ZONAB was later shown to be involved in regulation of proliferation epithelial cells (Balda et al., 2003). In paper II ZO-1 was used as a TJ marker to reveal the junctional complex that delimits the lumen in cultured thyroid follicles.

Adherens junction and E-cadherin

There are several types of adherens junctions (AJ), the one most studied in polarized epithelia is *zonula adherens* that encircles the cell completely like a belt at the apical/basolateral border located basal to the TJ (Farquhar and Palade, 1963). AJ consist of two protein complexes, the cadherin-catenin complex and the nectin-afadin complex. Both consist of a transmembrane adhesion molecule with an extracellular domain that interacts with corresponding molecules across the intercellular cleft and a group of cytoplasmic proteins that bind to the intracellular domain and connect it to the actin cytoskeleton. The superfamily of cadherins is responsible for calcium-dependent cell-cell adhesion and E-cadherin found in epithelial cells belongs the subfamily of classical cadherins (Nollet et al., 2000). Ca^{2+} binding to ectodomains in the

extracellular part of classical cadherins conveys a conformational change (Pokutta et al., 1994), which enables cis-dimerization between corresponding cadherin molecules on neighboring cells (Shapiro et al., 1995) and gradually builds up a structure resembling a zipper that provides strength to the AJ (Yap and Manley, 2001). The intracellular domain of E-cadherin binds to several members of the catenin family, i.e. p120 catenin ($p120^{ctn}$) (Reynolds et al., 1994), β-catenin and γ-catenin (Ozawa et al., 1989). Furthermore, α-catenin is also associated with the complex (Ozawa et al., 1989), although its interaction with E-cadherin is mediated by the binding to either β- or γ-catenin and thus not directly to E-cadherin itself (Aberle et al., 1994). β-Catenin is also involved in canonical Wnt signaling in which β-catenin translocates to the cell nucleus and trans-activates target genes involved in cell growth and survival (Valenta et al., 2012). The Wnt- β-catenin pathway may also regulate thyroid cells (Helmbrecht et al., 2001).

Epithelial to mesenchymal transition (EMT) is a fundamental biological process implicated in embryonic development, tissue repair and in association with tumor progression and metastasis (Kalluri and Weinberg, 2009). Loss of E-cadherin is a key feature and hallmark of EMT (Thiery, 2002). This aspect of E-cadherin was investigated along with observations of functional dedifferentiation in paper I of the thesis.

Desmosomes

Desmosomes comprise the third junction complex found in epithelial cells, although variants of this adhesive structure are shared by many other cell types. The adhesion molecules of desmosomes are membrane-spanning, cadherin-like proteins named desmocollins. Cytoplasmic proteins plakophilin and plakoglobin, the latter being identical to γ-catenin, link desmosomal cadherins to desmoplakin that in turn anchor the desmosome to the intermediate filaments of the cytoskeleton (Garrod and Chidgey, 2008). Very little is known of desmosomes in thyroid cells, although it is likely that they cooperate with the AJ in establishing a cohesive follicular epithelium.

Functional Properties

Thyroid cells are highly specialized cells needed for proper execution of the thyroid gland's functions. Conversely, loss of thyroid function leading to dedifferentiation is common in advanced thyroid cancer. In paper I, we were interested in studying thyroid dedifferentiation

in normal cells and the possibility of preventing or reverting the process by drug treatment that interferes with dedifferentiation signals inside the cells. To set the stage for the discussion of findings the next section will shortly summarize the most important elements of thyroid function and its normal regulation.

Iodide transport and NIS

Iodine is essential for normal thyroid function, being incorporated in the thyroid hormones. The anion species of iodine, iodide or I^-, is concentrated 40-fold or more in the thyroid due to an active transport mechanism originally named the “iodide pump” (Wolff, 1964). Studies on mice thyroid (Andros and Wollman, 1967), and later on in cultured cells using a bicameral culture model that enabled monitoring of polarized transport (Chambard et al., 1983; Nilsson et al., 1990) provided direct evidence that thyroidal iodide uptake occurs basolaterally, long before the molecular nature of the transporting protein was identified. In the same bicameral system it was also shown that transcellular transport of iodide depends on a second, apical efflux mechanism (Nilsson et al., 1990), hence, it is a two-step process.

In 1996, the sodium iodide symporter (NIS) responsible for the basolateral uptake was cloned and characterized from FRTL5 cells, a differentiated rat thyroid cell line (Dai et al., 1996) followed by cloning of the gene also in human (Smanik et al., 1996), mouse (Perron et al., 2001; Pinke et al., 2001) and pig thyroid cells (Selmi-Ruby et al., 2003). Notably, porcine NIS (pNIS) investigated in thesis paper I consists of two transcripts generated by alternative splicing instead of a single mRNA as in human, rat or mouse. The most abundant transcript of *pNIS* encodes a 643 amino acid protein with 85% identity to the human NIS. The reason for alternative splicing is not known (Selmi-Ruby et al., 2003). During NIS-mediated transport two sodium ions and one iodide ion are co-transported (Eskandari et al., 1997). The mechanism depends on Na^+/K^+ ATPase, also localized in the basolateral membrane (Gerard et al., 1985), which generates the driving sodium gradient. NIS is also capable of transporting several other anions that competitively may inhibit iodide uptake (Dohan et al., 2007). Loss of NIS expression is frequent in thyroid cancer and this will be discussed further in a later section.

While consensus prevails concerning NIS as the one and only basolateral iodide transporter, the identity of the apical transporter is more uncertain. A suggested candidate is the chloride

transporting protein pendrin, which is expressed apically in thyroid cells (Bidart et al., 2000; Royaux et al., 2000). Pendrin or SLC26A4 is encoded by Pendred syndrome (PDS) gene, and biallelic mutation of this gene causes Pendred syndrome, an autosomal recessive disorder characterized by deafness, goiter and a partial defect in iodine organification (Bizhanova and Kopp, 2011). Apical efflux is stimulated by thyroid stimulating hormone (TSH) (Nilsson et al., 1990), the main regulator of thyroid function, and it was recently shown that pendrin translocates to the membrane in response to TSH in PCCL3 rat thyroid cells suggesting a potential role in thyroid hormone synthesis (Pesce et al., 2012). However, since the majority of patients with Pendred syndrome either are euthyroid or have a mild hypothyroidism that may get worse only in iodine deficiency (Sato et al., 2001) it is likely that the apical efflux of iodide in thyroid cells is not only mediated by pendrin. Another proposed candidate is SLC5A8, also named human apical iodide transporter (hAIT) (Rodriguez et al., 2002), although it was later shown that this protein does not transport iodide (Paroder et al., 2006). Nevertheless, apical efflux is important to consider when evaluating cellular retention of radioiodine in experimental settings after tumor therapy with I-131.

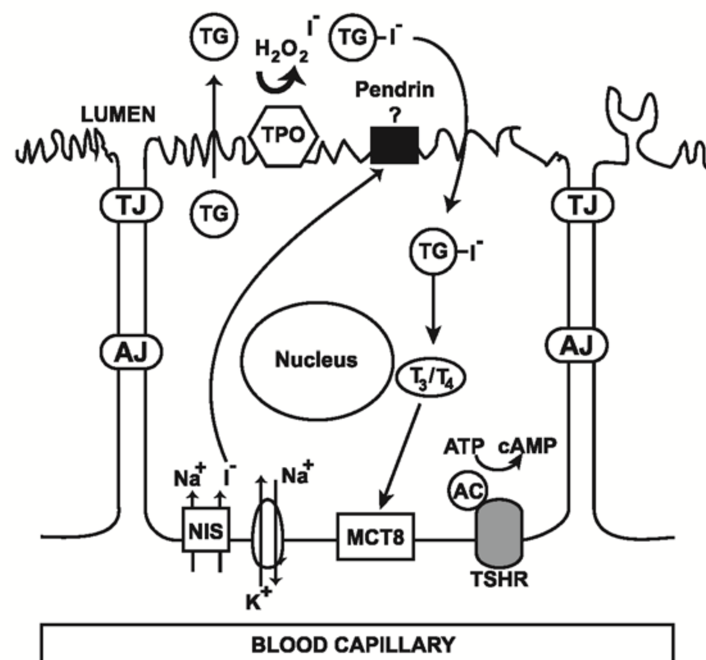

Fig.2. The polarized thyroid epithelial phenotype. Key molecules involved in different steps of thyroid hormonogenesis are differentially located in the basolateral and apical plasma membrane domains.

Thyroglobulin and thyroid hormone formation

Thyroid hormone biosynthesis is a complicated process that involves several independent steps and factors. In the following section the most important of these will be discussed. As already mentioned, TG can be thought of as a prohormone to T_3 and T_4. It is a very large dimeric glycoprotein (MW 660 kDa), the synthesis of which is governed by Nkx2-1 (formerly thyroid transcription factor-1 or TTF-1), Foxn1 (formerly thyroid transcription factor-2 or TTF-2) and Pax-8 that ensure thyroid specificity of expression (Damante and Di Lauro, 1994; Zannini et al., 1997). After synthesis of the peptide chains in the endoplasmic reticulum and glycosylation in Golgi, mature but yet un-iodinated TG is transported in vesicles to the apical cell surface where it is released into the follicle lumen by exocytosis. Iodination of TG is catalyzed by thyroperoxidase (TPO) located in the apical membrane facing the lumen. TPO converts I^- to an oxidized iodine species that covalently binds to tyrosyl residues in TG thus producing 3-iodotyrosine (MIT) and 3,5´-diiodotyrosine (DIT). In the following coupling reactions, also requiring TPO activity, T_4 and T_3 are formed from two DIT or one DIT and one MIT, respectively (Dunn and Dunn, 2001; Ekholm, 1990). The oxidation reactions require H_2O_2 that is produced by dual oxidase I and 2 (DUOX1 and DUOX2) also presented in the apical membrane (De Deken et al., 2002; Dupuy et al., 1991). Since H_2O_2 is potentially cytotoxic there is a need of a protective mechanism that degrades excess H_2O_2. The intracellular level of H_2O_2 is kept low by glutathione peroxidase which also prevents intracellular iodination (Ekholm and Bjorkman, 1997). In addition, thioredoxin reductase has also been suggested to regulate intracellular H_2O_2 and prevent H_2O_2-induced apoptosis (Kim et al., 2000).

TG with its iodo-amino acid residues incorporated in the peptide chains is stored in the lumen until there is a need of hormone release. This process starts with internalization of TG executed by both micropinocytic vesicles and macropinocytosis, the latter through the formation of so called pseudopods that project from the apical cell surface in to the colloid (Ericson, 1981). Internalized TG can take two different pathways. The most prominent route involves fusion of TG-containing vesicles with early endosomes followed by proteolysis in secondary lysosomes (Bernier-Valentin et al., 1990). T_4 may be deiodinated to T_3 already in the thyroid. However, most T_4 enters the blood stream and will be converted to T_3 in peripheral tissues (Chanoine et al., 1993). The mechanism by which free thyroid hormones are released from the cytoplasm is not fully understood but mononocarboxylate transporter 8 (MCT8), originally identified as a specific thyroid hormone transporter in target organs as

liver, kidney, brain and heart (Friesema et al., 2003) was recently found to be involved in the export of thyroid hormones from the thyroid gland in mice (Di Cosmo et al., 2010). The second mechanism of TG transport is the direct basolateral release of intact TG in a process called transcytosis and this probably explains the presence of circulating TG in blood (Romagnoli and Herzog, 1991). The turnover of TG under the influence of dedifferentiation stimuli (epidermal growth factor) was evaluated in paper I.

Differentiation signals

TSH

TSH or thyrotropin is the main regulator of thyroid function. It is secreted from the anterior pituitary gland in response to thyrotropin-releasing hormone (TRH) produced in the hypothalamus (Persani, 1998) and regulated by a negative feedback mechanism through circulating thyroid hormones. Thus, a hypothyroid state reactively leads to increasing TSH levels that stimulate the gland even more. This effect involves many aspects of thyroid function collectively contributing to increased hormone biosynthesis. One of the first characterized TSH-regulated functions was iodide trapping *in vivo* (Halmi, 1954). It is now known that an increased expression of NIS is primarily responsible for this effect as shown both *in vitro* and in animal models (Levy et al., 1997; Saito et al., 1997). In addition, TSH stimulates the expression of TG (Roger et al., 1985), apical efflux of iodide (Nilsson et al., 1990), iodination (Ekholm and Wollman, 1975), internalization of iodinated TG (Ericson, 1981) and thyroid hormone release (Dumont et al., 1971).

TSH acts by binding to the TSH receptor (TSHR) present in the basolateral membrane of the follicle cells (Chambard et al., 1983). TSHR is a G protein-coupled receptor (Libert et al., 1989) that signals through activation of both G_s and G_q (Allgeier et al., 1994). G_s stimulates adenylate cyclase (AC) that will increase 3´5´-cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA) (Dumont et al., 1971). G_q stimulates phospholipas C (PLC) (Jhon et al., 1993), which stimulates hydrolysis of phosphoinositide to inositol triphosphate (IP_3) and diacylglycerol (DAG) that increase the concentration of intracellular Ca^{2+} and activates protein kinase C, respectively. Significant species specific differences have been pointed out when it comes to the underlying signaling mechanisms of the TSH response in thyrocytes (Song et al., 2010). For example, in dog cells TSH stimulates activation of the cAMP pathway leading to H_2O_2 production while this occurs through Ca^{2+}–DAG signaling in human or pig cells (Song et al., 2010).

TSH stimulates thyroid cell proliferation most evidently resulting in goiter development in conditions where circulation TSH is chronically elevated (in hypothyroidism) or in the presence of stimulating autoantibodies against TSHR (in Graves' disease). TSH can function as a mitogen either through cAMP pathway (Deleu et al., 1999; Dremier et al., 2002) or indirectly through its permissive action on peptide growth factors (Kimura et al., 2001). In pig thyroid cells used in this thesis (papers I and II), TSH is not mitogenic (Gartner et al., 1985).

TSH was previously shown to promote the epithelial integrity of porcine thyrocytes when Ca^{2+}-dependent cell-cell adhesion was abrogated (Nilsson et al., 1991). The mechanism involves stabilization of E-cadherin binding that prevents its premature degradation (Larsson et al., 2004), although TSH may also stimulate the expression of E-cadherin at the transcriptional level (Brabant et al., 1995). Thus, TSH appears to be required to establish firm adhesion between thyroid epithelial cells and that this probably is important to secure cohesiveness of the follicular wall and prevent unwanted leakage of luminal content. TSH stimulation was, therefore, routinely used in the investigation of growth factor effects in this thesis work.

IGF-1

Insulin and insulin like growth factor-1 (IGF-1) exert moderate proliferative effects that are permissive to the action of TSH in human thyrocytes (Roger et al., 1988). The need of concomitant signaling of TSH and the IGF-1 signaling pathway for goiter formation was recently shown in mice with conditional deletion of the IGF-I receptor (IGF-1R) (Ock et al., 2013). However, over-expression of IGF-1, IGF-R or both, increased thyroidal iodide uptake while at the same time circulating TSH levels decreased, indicating that IGF-1 promotes thyroid function *in vivo* (Clement et al., 2001). Earlier studies on pig thyroid cells showed that IGF-1 in the absence of TSH stimulates only mildly iodide transport whereas in its presence iodide transport is highly potentiated (Ericson and Nilsson, 1996).

IGF-IR is a heterotetramer consisting of two ligand binding alfa subunits being completely extracellular and two transmembrane beta subunits each containing a tyrosine kinase domain in the cytoplasmic portion. After ligand binding, the activated receptor is autophosphorylated leading to phosphorylation of several target proteins of which insulin receptor substrate-1 and 2 (IRS-1 and 2), which function as docking sites for SH-2 containing proteins such as PI3K, are of particular importance. Phosphorylated IRS-1 also acts as a docking site for Grb-2,

which upon activation binds RAS and initiates MAPK signaling. However, IGF-1R can influence multiple intracellular pathways that partly explain the many functions of IGF-1 in cells and tissues (LeRoith et al., 1995). IGF-1 was not used in this thesis, but fetal serum contains significant amounts of IGF-1 which makes it a relevant molecule to consider.

Dedifferentiation signals

EGF

Epidermal growth factor (EGF), one of the most well-studied peptide growth factors ever, was first isolated from mouse submaxillary gland and found to have a stimulatory effect on the proliferation of epidermal keratinocytes (Cohen, 1962; Cohen and Elliott, 1963). EGF is a 53 amino acid protein that belongs to a family of growth factors that also includes transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), betacellulin, amphiregulin, neuregulin, epigen and epiregulin. These proteins are ligands to members of the EGF receptor (EGFR) or ErbB receptor family comprising, apart from EGFR, erbB2/Her2, erbB3/Her3 and erbB4/Her, all of which share a common structure with an extracellular ligand-binding domain and an intracellular receptor tyrosine kinase (RTK) domain. EGF binding triggers EGFR homo- or heterodimerization and autophosphorylation, which is mediated by the RTK domain that will also function as docking site for different proteins further down in the signaling pathway, (Burgess, 2008). This will be described in more detail in a separate section below.

In the thyroid, the mitogenic effect of EGF was first shown in sheep (Westermark and Westermark, 1982) and later confirmed in other species as dog, pig and human. Besides causing stimulation of thyroid cell proliferation, EGF is a powerful antagonist to TSH-stimulated thyroid function including down-regulation of TG and TPO expression (Kasai et al., 1989; Pratt et al., 1989; Roger et al., 1985) and loss of iodide trapping capacity (Bourke et al., 1991; Pratt et al., 1989; Waters et al., 1987). Cultured in presence of EGF, dog thyrocytes lose both the responsiveness to TSH and cAMP-mediated stimulation of proliferation (Roger et al., 1992). Together, this argues that EGF is a major dedifferentiation factor with potential implications in the pathophysiology of thyroid proliferative diseases as hypothyroid goiter (Pedrinola et al., 2001) and thyroid cancer (Knauf, 2011). In fact, stimulation of human thyrocytes with EGF in the presence of serum confers a profound change in global gene expression that mimics the expression profile found in papillary thyroid cancer (Hebrant et al., 2007). EGF also promotes the development of EMT elicited by TGF-beta in primary porcine

thyroid cells (Grande et al., 2002). It should be noted, however, that in the absence of other EMT inducers, EGF treatment does not seriously inflict on the thyroid epithelial phenotype, despite a strong stimulation of cell proliferation and migration. For example, porcine thyroid cells cultured in a collagen matrix form new follicles when stimulated with EGF (Westermark et al., 1991) and in monolayer cultures in bicameral chambers the epithelial barrier is preserved, although at the same time, TSH-stimulated iodide transport is repressed by EGF (Nilsson and Ericson, 1994). In papers I and II of this thesis EGF was used to dedifferentiate both functionally and structurally pig thyroid cells in 2D and 3D culture, which was further investigated for the potential use of small molecule kinase inhibitors against key components of EGFR signaling pathways to prevent the effect.

TGF-beta

Transforming growth factor beta (TGF-β) belongs to a superfamily of cytokines involved in many different cellular processes implicated in growth, differentiation and survival of various cell types (Heldin et al., 1997). TGF-β consists of three different isoforms, TGF-β1 (Derynck et al., 1985) TGF-β2 (de Martin et al., 1987) and TGF-β3 (Derynck et al., 1988) of which TGF-β1 is mostly studied. A common feature of TGF-β family receptors is signaling through a serine/threonine kinase domain. TGF-β binds to type II receptor (TβR-II) that recruits and phosphorylates the type I receptor (TβR-I) (Wrana et al., 1994). The major signaling pathway of the activated TGF-β receptor involves SMAD proteins that are stimulated to enter the nucleus and after formation of a complex with co-repressors or co-activators gene expression is either turned on or off (Massague, 2000). TGF-β stimulation of normal epithelial cells causes growth inhibition and this is also true for thyroid cells (Taton et al., 1993). The pleiotropic effects of TGF-β signaling aside of growth regulation are very diverse and depend on the cell type and the context. Growth arrest and induction of apoptosis are responsible for the tumor suppressive effects of TGF-β (Inman, 2011). However, TGF-β also has tumor promoting effects in advanced cancer and is one of the most powerful stimuli of EMT typically manifested by loss of E-cadherin expression and acquirement of motile phenotype (Heldin et al., 2012). EMT makes the tumor cells invade and metastasize, hallmarks of disseminated cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

As already mentioned, in pig thyroid cells TGF-β1 in synergy with EGF, induces EMT leading to loss of epithelial integrity, loss of E-cadherin and gain of N-cadherin expression

(Grande et al., 2002). TGF-β was not investigated directly but its involvement in EMT makes it relevant to mention.

MAPK Signaling Pathway

Further interest in EGF-induced growth and dedifferentiation is due to its major downstream mitogen-activated protein kinase (MAPK) signaling pathway, which comprises several proto-oncogenes and is constitutively activated in many malignant tumors. The canonical MAPK pathway consists of four cascades classified according to the last protein in each arm, that is extracellular signal-related kinases 1 and 2 (ERK1/2) , c-jun N-terminal kinase (JNK) 1, 2 and 3, p38-MAPK and ERK5. Peptide growth factor receptors are mainly regulators of the ERK1/2 cascade whereas JNK and p38-MAPK is activated by different cellular stress stimuli, but there is also evidence of crosstalks between the different MAPK pathways (Pritchard and Hayward, 2013). All four cascades consist of a central core of three kinases being activated in sequence: MAPK kinase kinase (MKKK), MAPK kinase (MKK) and MAPK. Thus, in a series of amplifying threonine and tyrosine phosphorylations MKKK activates MKK that in turn triggers the effector kinase, ERK1/2 in the case of EGFR signaling (Yang et al., 2013). The identity of the kinases in the linear signaling pathway is unique for each cascade (Pritchard and Hayward, 2013). In this thesis particular interest is focused on the MAPK pathway downstream of EGFR, which will be described in more detail.

A systematic study of the phosphotyrosine interactome demonstrated that EGFR has several different binding partners including growth factor receptor binding protein 2 (Grb2). Grb2 contains Src homology 2 (SH2) and 3 (SH3) domains that provide a link between the receptor and the guanine nucleotide exchanges factors (GEFs) i.e. son of sevenless homologue 1 and 2 (SOS1, SOS2) (Lowenstein et al., 1992). Guanosine triphosphatases (GTPases), RAS in the case of ERK1/2 pathway, play a crucial role in signal transduction. When bound to guanosine diphosphate (GDP) the GTPase is inactive but with the assistance of GEFs, GDP dissociates from the GTPase allowing the binding of guanosine triphosphate (GTP) by which the GTPase is activated. Further on, the GTPase enters an inactive state through hydrolysation of GTP to GDP which is facilitated by guanosine activating proteins (GAPs) (Cherfils and Zeghouf, 2013).

The RAS family of small GTPases consists of three isoforms: HRAS, KRAS and NRAS. Upon activation RAS proteins interact with various effectors including PI3K, Af6, PKCζ and RAF, which takes part in many cellular processes including growth, survival and migration (Rajalingam et al., 2007). MKKK activated by RAS also consists of three proteins, ARAF, BRAF and CRAF of which BRAF is most easily activated and also has a higher basal kinase activity than the other two members of the family (Wellbrock et al., 2004). All three RAFs can activate MKK, the MEK1/2 kinases, which are the only widely accepted RAF substrate (Matallanas et al., 2011). ERK1/2 in turn is the only known substrate MEK1/2. However, after this point the signaling cascade diverges to many different effector mechanisms as illustrated by the fact that more than 160 substrates to ERK1/2 exist (Yoon and Seger, 2006) and even more candidates have been suggested (Courcelles et al., 2013). ERK1/2 substrates include nuclear targets (e.g c-fos and c-jun), substrates belonging to the ribosomal S6 kinase (RSK) family and cytoskeletal proteins (e.g paxillin). There are also MAPK phosphatases with the potential to dephosphorylate and thereby modulate the amplitude and duration of MAPK signaling. These can either be specific to tyrosine, serine or threonine or possess a dual specificity for both serine and threonine (Roskoski, 2012). MEK inhibition and evaluation of its consequences were a central theme in all papers of this thesis.

PI3K/AKT Signaling Pathway

Another signaling pathway downstream of EGFR is the phosphoinositide-3 kinase (PI3K)/AKT pathway. There are three classes of PI3Ks of which class IA is the most extensively studied. PI3Ks are heterodimers classically composed of a regulatory subunit, p85, comprising five isoforms and a catalytic subunit of which there are three subunits, p110α, p110β and p110δ. The regulatory p85 can bind directly to RTK through the SH2 domain by which PI3K is activated and also translocated to the plasma membrane (Vanhaesebroeck et al., 2012). In addition, PI3K is also a direct substrate to RAS (Sjolander et al., 1991). When activated, class I PI3Ks phosphorylates the inositol ring on the membrane lipid phosphatidylinositol-4-5-bisphosphate ($PI(4,5)P_2$). When converted to a phosphatidylinositol-3-4-5-triphosphate ($PI(3,4,5)P_3$) this provides a binding site for downstreams signaling proteins which contains a so called pleckstrin homology (PH) domain. Two important proteins with a PH domain are AKT, also called protein kinase B (PKB), and phosphoinositide-dependent kinase 1 (PDK1) (Cantley, 2002). Full activation of AKT requires phosphorylation on two sites, threonine 308 (T308) by PDK1 and serine 473 (S473)

by mammalian target of rapamycin (mTOR) (Sarbassov et al., 2005). Termination of PI3K signaling through degradation of PI(3,4,5)P_3 is mediated by phosphatases of which the most important is phosphatase and tensin homolog (PTEN). Dephosphorylation of the 3 position of PI(3,4,5)P_3 by PTEN inactivates AKT and downstream signaling of the pathway (Maehama and Dixon, 1998). Many substrates have been identified downstream of AKT and the pathway appears to involve even more cellular functions than the MAPK pathway, ranging from vital processes in cell metabolism to differentiation of specialized tissues in development also comprising growth and migration. Of particular relevance for the interpretation of data presented in paper II is the role of PI3Ks in epithelial morphogenesis and establishment of epithelial junctions (Rivard, 2009; Shewan et al., 2011).

Thyroid Cancer

Thyroid cancer is the most common endocrine malignancy after ovarian cancer representing approximately 1% of all malignant tumors. As for other diseases of the thyroid gland cancer is more frequent in females, for example, in Sweden 2011 71% of all newly diagnosed cases were women (Socialstyrelsen, 2013). Thyroid cancer is divided in several subtypes depending on the histopathological diagnosis. The most common tumor constituting 80-85% of all thyroid malignancies is papillary thyroid cancer (PTC) derived from follicular cells. PTC together with follicular thyroid cancer (FTC) is collectively called differentiated thyroid cancers (DTC). Poorly differentiated thyroid cancers (PDTC) usually arises by tumor progression of PTC. Anaplastic thyroid cancer (ATC) is rare but one of the most aggressive tumors of all in man. Tumor spreading characteristics vary depending on subtype. PTC is subjected to lymphogenic spread to regional lymph nodes in the neck while FTC more often gives rise to distant metastases as in lung, skeleton and brain through hematogenic dissemination. PDTC mostly derived from advanced PTC is locally aggressive with an invasive growth. ATC is highly invasive often with engagement of the trachea or surrounding anatomic structures in the neck and distant metastases are found early (Xing, 2013). Patients suffering from DTC have mostly a very good prognosis and the overall 5-years survival may be as high as 97% (Howlader N). In comparison, ATC is very lethal with a median survival of 5 month and a 1-year survival of less than 20% (Smallridge and Copland, 2010). Treatment of DTC includes thyroidectomy followed by radioiodine therapy (iodine-131), a therapy taking advantage of the natural iodide handling system in the thyrocytes. Hence, PDTC that have lost the capacity of transport and trapping of iodide are refractory to radioiodine

treatment and few alternatives of adjuvant treatment exist for this group of patients. Medullary thyroid cancer (MTC) derived from C cells is a neuroendocrine tumor. MTC is not a subject in this thesis and will not be further commented on.

Genetic alterations in thyroid cancer

Several oncogenic alterations in genes encoding key molecules in growth-promoting signaling pathways are described for the distinct entities of thyroid cancer. In addition, inactivation of tumor suppressor genes is implicated in tumor progression. The most important of these will be briefly described in the following section.

RET/PTC rearrangements

Approximately 20-40% of sporadic of PTC harbor *RET/PTC* rearrangements, a genetic alteration unique for PTC and caused by fusion of the proto-oncogene *RET* with a partner gene, the identity of which determines further subtyping of the tumor (Fusco and Santoro, 2007). *RET/PTC1* and *RET/PTC3* are most prevalent. *RET/PTC3* predominates in the cohort of children with radiation-induced thyroid cancer appearing after the Chernobyl nuclear plant accident in 1986 (Nikiforov et al., 1997). The tyrosine kinase portion of RET that convey the oncogenic signal. The fusion protein dimerizes independently of ligand binding leading to autophoshorylation and formation of docking sites for molecules initiating MAPK signaling and in fact PI3K pathway can also be activated (Riesco-Eizaguirre and Santisteban, 2007). RET is not expressed in normal thyroid follicular cells. However, the development of thyroid C cells requires RET and MTC can also arise from activating *RET* mutations.

$BRAF^{V600E}$ mutation

A valine-to-glutamate substitution at residue 600 in BRAF is the most common activating *BRAF* mutation in human cancer, being most prevalent in melanoma and colon carcinoma (Davies et al., 2002). $BRAF^{V600E}$ is also found in approximately 45% of PTC (Xing, 2013). The mutation leads to constitutive activation of the MAPK pathway and increased phosphorylation of ERK that promotes the proliferation of tumor cells. Mutated *BRAF* is overrepresented in PDTC and ATC derived from PTC (Nikiforova et al., 2003) and has been correlated to a poorer clinical prognosis (Xing et al., 2005). Inhibitors specific to mutant BRAF are initially efficient in targeted therapy of melanoma but are also prone to elicit drug resistance (Lito et al., 2013). Since $BRAF^{V600E}$ down-regulates the expression of several genes involved in thyroid hormone synthesis including *NIS*, *TPO* and *TG* (Durante et al., 2007;

Mian et al., 2008; Romei et al., 2008), specific inhibitors to mutant BRAF might be useful in restoring iodide transport capacity and improve the therapeutic outcome of radioiodine treatment. $BRAF^{V600E}$ inhibitors have also been given to patients with metastatic PTC with promising results and a phase II study has also been initiated (Kim et al., 2013).

RAS mutations

Another common alteration is *RAS* mutations for which the encoded GTPase is constantly bound to GTP and therefore in constitutively active. *NRAS* mutations are frequent in FTC, PDTC and ATC (Xing, 2013). Since both RAF and PI3K are effectors downstream of RAS, either pathway can potentially contribute to tumorigenesis from *RAS*-mutated cells (Malumbres and Barbacid, 2003). A predominant role of AKT has been suggested for FTC whereas increased phosphorylation of both ERK1/2 and AKT often coexists in ATC, suggesting that targeted therapy of both pathways could be more efficient in these patients (Liu et al., 2008).

EGFR amplification

The presence of activating *EGFR* mutations in tumors have led to the development of EGFR tyrosine kinase inhibitors such as gefitenib currently used in patients with $EGFR^{CA}$ positive non-small cell lung carcinoma with some benefits in delaying disease progression (Lee et al., 2013). *EGFR* mutations are rare in thyroid cancer (Ricarte-Filho et al., 2012). However, copy number gain of *EGFR* has been reported for 30-40% of FTC and ATC (Liu et al., 2008) with over-expression mainly observed in dedifferentiated thyroid tumors (Landriscina et al., 2011).

PI3KCA mutations

The *PI3KCA* gene encodes for the p110α subunit and activating mutations or copy number gain leading to increased PI3K/AKT signaling have been reported in thyroid cancer with highest frequency found in FTCs (5-15%) or ATCs (15-25%) (Xing, 2013).

PTEN alterations

Deletion or inactivating mutations of *PTEN* that negatively regulates AKT in normal cells will lead to increased activity of the PI3K/AKT pathway and promotion of tumor development (Xing, 2013). *PTEN* is also epigenetically regulated and higher levels of methylated *PTEN* coexisting with other PI3K/AKT alterations have been reported for FTC and PTC (Hou et al., 2008).

Pax8-PPARγ

Translocation between chromosome 2 and 3 gives rise to the Pax8-peroxisome proliferator activated receptor γ (PPARγ) fusion gene (*PAX-PPARγ*). This fusion gene encodes for a fusion protein that acts as a dominant negative inhibitor of wild type PPARγ (Kroll et al., 2000) and is found in up to 60% of FTC (Xing, 2013).

TP53 mutations

Inactivating mutations in the tumor suppressor *TP53* are preferentially found in advanced and highly malignant tumors indicating an important role in tumor progression (Malaguarnera et al., 2007). Mutated p53 occurs in 25% of PDTC and the majority (70-80%) of ATC (Xing, 2013).

Small Molecule Kinase Inhibitors

Clarifying the identity of which kinase is overactive leading to dysregulated pathway signaling in cancer, opens the opportunity for targeted treatment, by allowing the development of specific inhibitors. Currently in Sweden only the RTK inhibitor vandetanib is approved for treatment of metastatic MTC and in US cabozantinib is in addition approved since last year for the same indication. However, clinical trials with small molecule kinase inhibitors for other forms of thyroid cancer are in progress (Xing, 2013). This thesis makes use of three established kinase inhibitors to block MEK, PI3K and mutant BRAF, respectively, in cultured normal thyrocytes and thyroid cancer cell lines. It is therefore appropriate to describe their pharmacological feature in some more detail.

U0126 directed against MEK

Synthesized in the late 1950's 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene or U0126 (Middleton et al., 1958) was in 1998 identified as a specific MEK1/2 inhibitor (Favata et al., 1998). The drug was already known to inhibit gene activation through transcription factor AP-1 involved in cell cycle control (Angel and Karin, 1991). U0126 inhibits MEK1/2 non-competitively by binding to the enzyme on a position different from the binding sites of ATP or ERK (Favata et al., 1998). It is regarded as one of the most powerful pan-MEK inhibitors. However, due to pharmaceutical limitations it cannot be used clinically, although there are other substances MEK inhibitors with better profiles concerning bioavailability and solubility are available today (Fremin and Meloche, 2010). Nevertheless,

U0126 is widely used experimentally as an important tool to evaluate the contribution of MAPK signaling.

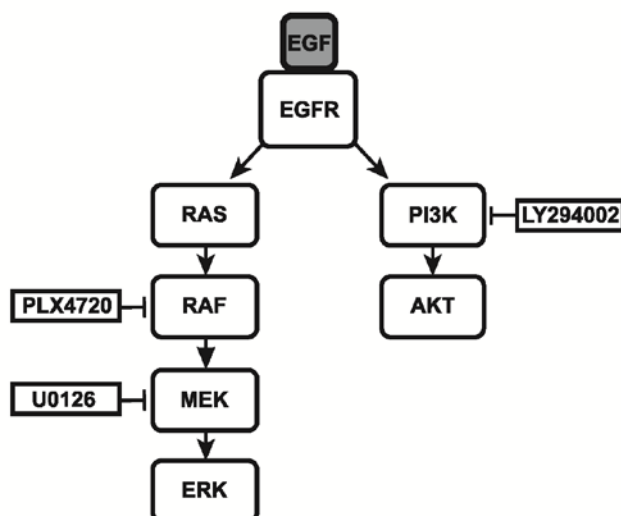

Fig.3. Signaling pathways showing the key points of action of the major inhibitors used in this study.

LY294002 directed against PI3K

The natural agent wortmannin originally found to inhibit PI3K is unspecific and afflicted by many off-target effects. Another PI3K antagonist, the flavonoid quercetin, was used as a model to synthesize more selective inhibitors to PI3K. One of them, (2-(4-morpholinyl)-8-phenylchromon(e2 -morpholino-8-phenyl-4H-l-benzopyran-4-one) or LY294002 inhibits PI3K with a much higher specificity and potency as compared to quercetin (Vlahos et al., 1994). LY294002 is a pan-PI3K inhibitor acting competitively by blocking ATP binding to all PI3K isoforms at micromolar range. However, the specificity profile of LY294002 has been reported to be broader than first expected (Gharbi et al., 2007). For example, the catalytic site of p100α and mTOR is structurally similar explaining the cross-reactivity and proposing an advantage that LY294002 and similar drugs may be used as a dual PI3K/mTOR inhibitor (Markman et al., 2010). Hence, the possibility of multiple drug effects should be considered when interpreting experimental data using LY294002.

PLX4720 directed against mutated BRAF

The high prevalence of $BRAF^{V600E}$ mutations in human cancers has encouraged the development of inhibitors specifically targeting the mutated form of the kinase. N-(3-(5-chloro-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluorophenyl)propane-1-sulfonamide

or PLX4720 belongs to the first generation of inhibitors that binds competitively to the ATP-binding pocket in the kinase-active conformation and efficiently reduces the growth for both melanoma cell line and melanoma tumor xenografts (Tsai et al., 2008). PLX4032 or vemurafenib is an analogue to PLX4720 but with more favorable pharmacokinetic properties in animal studies (Bollag et al., 2010), which led to the recent approval of vemurafenib for treatment of metastatic melanoma. Unfortunately, efficient treatment with BRAF inhibitors is limited by the high prevalence of acquired drug resistance. Several mechanisms including amplification of wild type BRAF (or CRAF), appearance of spliced versions of BRAF being drug insensitive, or additional mutations downstream of RAF have been identified (Lito et al., 2013). Interestingly, there is a divergence in drug response between BRAF mutant cancer cell lines in that thyroid-derived and but not melanoma-derived cell lines reactivate MAPK signaling pathway by respond to PLX4032 treatment with accelerated HER3 signaling leading to drug resistance (Montero-Conde et al., 2013).

Experimental Models of Thyroid Cancer

Many experimental systems have been developed to model tumor biology. All of them, with their pros and cons, are used in order to increase the understanding of uncontrolled cell proliferation and tumor growth, migration and local invasion of tumor cells, and the metastasizing process eventually leading to disseminated cancer. Experimental testing of potential anti-cancer drugs aiming to affect these parameters is of course a very important task in search for new treatments in patients with relapsing or metastatic disease. The next section will briefly highlight different *in vivo* and *in vitro* models previously employed in thyroid cancer research.

Genetically modified mice

It is well known that rodents chronically stimulated with TSH (Wynford-Thomas et al., 1982), or are exposed to ionized radiation or carcinogens develop thyroid tumors (Al-Hindawi et al., 1977; Kitahori et al., 1988; Wollman, 1963; Wollman and Reed, 1963). However, with the invention of targeted expression in transgenic mice it was possible to investigate thyroid tumorogenesis elicited by a single known oncogene (Ledent et al., 1991; Ledent et al., 1995; Ledent et al., 1994). Later on, the discovery of specific oncogenic mutations in thyroid cancer initiated generation of mouse models in which over-expression of the mutated gene elicited thyroid tumors with similar properties as the human counterpart. There are several oncogenic

mouse models available to this date (Kim and Zhu, 2009; Russo et al., 2011). Among these RET/PTC1 (Santoro et al., 1996), RET/PTC3 (Jhiang et al., 1998; Powell et al., 1998) and $BRAF^{V600E}$ (Knauf et al., 2005) nicely recapitulates the typical PTC variants. Thyroid-specific $BRAF^{V600E}$ expression further leads to an invasive tumor and progression of the phenotype resembling EMT associated with TGF-β production (Knauf et al., 2005). Interestingly, in a doxycyclin-inducible mouse model there are indications of reversibility and tumor regression of BRAF-induced PTC when the oncogenic signal is discontinued (Chakravarty et al., 2011). In the same model, treatment with small molecule kinase inhibitors inhibiting $BRAF^{V600E}$ or MEK has a therapeutic effect on tumor growth and which in addition reverses dedifferentiation and at least partly recovers radioiodine uptake (Chakravarty et al., 2011). Presently, there is only one proposed ATC mouse model reported, co-inactivation of two tumor suppressor genes *TP53* and *PTEN*, suggesting involvement of PI3K pathway in the tumorigenesis of ATC (Antico Arciuch et al., 2011).

Cell lines

Another and perhaps more convenient way to experimentally study oncogenes and tumor-associated factors is the use of immortalized but untransformed non-human cell lines transfected with the gene of interest. For example, inducible $BRAF^{V600E}$ expressed in the PCC13 rat thyroid cell line makes a model of tumor progression in which TGFβ up-regulated by $BRAF^{V600E}$ induces EMT in cooperation with constitutive MAPK signaling (Riesco-Eizaguirre et al., 2009). Otherwise, cancer cell lines established from malignant thyroid tumors have served as valuable tools in innumerable studies aiming to study various aspects of tumor cell behavior with the benefit of being derived from human tumors. However, significant redundancy and misidentification were recently found among several thyroid cancer cell lines (Schweppe et al., 2008). The cell lines analyzed in this study are summarized in table 1. Thus, choice of cell line should be carefully considered before entering a study in which cell origin and context are of importance. Another concern regarding cell lines is to be aware of the apparent risk of altered cell behavior during propagation due to selection and acquisition of new mutations. It was recently shown that cell lines established from DTC have adopted an *in vitro* signature with a gene expression profile and phenotype that closest resemble undifferentiated tumors (van Staveren et al., 2007). It is likely that such changes inflict on the responsiveness to reagents and drugs that makes comparison to the originating primary tumor and translations to *in vivo* conditions difficult or even impossible. Nevertheless, some progress have been made using cell lines to better understand mechanisms

of functional dedifferentiation that might explain loss of NIS expression and resistance to radioiodine therapy, trying to redifferentiate and recover NIS-mediated iodide uptake. For example by targeting established thyroid cancer cell lines with poor iodide uptake with small molecule kinase inhibitors alone or in combination with histonedeacetylase (HDAC) inhibitor SAHA increased trapping of radioiodide was observed (Hou et al., 2010). In addition, DNA methylation inhibitors have been shown to partially restore both *NIS* expression and iodide uptake (Venkataraman et al., 1999). On the other hand, another DNA methylation inhibitor (5-aza-2'-deoxycytidine) failed to redifferentiate thyroid cancer cell lines in a recent study (Dom et al., 2013). Contradicting results are also reported for all-transretinoic acid (ATRA) used for the same purpose (Malehmir et al., 2012; Vivaldi et al., 2009) .

Primary cell culture

Tumor cells are by definition fundamentally different from normal cells in particular regarding growth control. However, differentiated tumors retain many features of the ancestral cell. Moreover, growth factor stimulation of normal epithelial cells can induce not only proliferation but also dedifferentiation, cell migration and even complete EMT suggesting primary cultures may be instrumental to better understand at least some aspects of tumor cell behavior shared by non-transformed cells. Tumor cells produce their own growth factors, which is important for sustained proliferative signaling (Hanahan and Weinberg, 2011). Auto- or paracrine growth factors may also be important for induction of EMT and tumor cell migration. As mentioned before, the gene expression profile of normal human thyrocytes treated with EGF in the presence of serum mimics the transcriptome monitored in PTC (Hebrant et al., 2007). This included, for example galectin-3, which is not expressed in the normal thyroid but potentially can transform thyrocytes (Takenaka et al., 2003).

Combined activation by EGF and TGF-β induces EMT in normal porcine thyrocytes (Grande et al., 2002) and matrix invasion of cells from 3D cultured thyroid follicles (Nilsson et al., 1995). Together this indicates that primary normal thyroid cells under the influence of growth factors can serve as a potentially interesting model for further investigation of targeted drug effects.

Studies on primary human thyroid tumor samples in cell culture or explants are yet few, but recent findings suggest this is a promising future approach for possible individualized patient investigations (Antonelli et al., 2008a; Antonelli et al., 2008b; Bravo et al., 2013).

Aims of the Thesis

Paper I: Recovery of NIS expression by inhibition of MEK

Possibilities to redifferentiate thyroid cancer cells and obtain sufficient expression of NIS, the primary iodide transporter in normal thyroid cells, in order to improve radioiodine therapy have proven to be difficult for various reasons. There is also a relative lack of knowledge of mechanisms that repress NIS expression leading to loss of thyroid iodide uptake, although most likely both genetic and epigenetic aberrations cooperate. This study was undertaken to elucidate if we can learn more from investigations on normal thyrocytes triggered to dedifferentiate by EGF-induced activation of the MAPK signaling pathway. Specifically, identification of limiting factors or culture conditions for the recovery of NIS expression by single drug treatment with MEK inhibitor was addressed. To this purpose 2D and 3D cultures were compared based on reports that microenvironment influences gene expression and drug responses in both normal and tumor cells (Bissell and Hines, 2011; Bissell et al., 2003; Correia and Bissell, 2012)

Paper II: Inhibition of thyroid cell migration by MEK and PI3K inhibitors

Experimental findings mainly based from non-thyroid studies indicate that uncontrolled PI3K/AKT signaling play a major role in cancer cells migration and invasiveness and that PI3K inhibition may diminish tumor spreading. However, co-activation of other oncogenic pathways may modify the drug response and even lead to the opposite, and increased metastatic behavior, as recently reported for colon cancer (Tenbaum et al., 2012). In thyroid there are only few studies so far addressing to role of PI3K in tumor cells (Burrows et al., 2013), and knowledge of the response of normal thyroid cells to PI3K inhibitors is sparse. This was investigated in 3D-cultured thyroid follicles focusing on the contribution of PI3K in EGF-induced cell migration and any possible influences on preservation of the epithelial phenotype.

Paper III: Inhibition of thyroid cancer cell invasion by BRAF^{V600E} and MEK inhibitors

Scratch wounding and transmigration across matrix-coated permeable filter are convenient but simplified methods for investigation tumor cell migration and invasion *in vitro*. In this study we wanted to investigate whether 3D culture inside extracellular matrix modifies the migrating behavior of PTC- and ATC-derived thyroid cancer cell lines harboring oncogenic

BRAF^{V600E} mutations and the response to a BRAF-specific inhibitor. The possibility of rebound activation of MAPK signaling during prolonged exposure to BRAF inhibitor and its consequences for invasiveness and the responsiveness to co-treatment with a MEK inhibitor were also addressed.

Methodological Considerations

In the following section some general aspects of the methods used in this thesis and related issues of concern will be discussed. For more information the reader is referred to the individual papers.

Isolation of porcine thyroid follicles

Major parts of this thesis (paper I and II) are based experiments on primary cultured porcine thyrocytes. Isolation and enrichment of porcine thyroid follicles is a well established method in our laboratory developed already in 1980. Basically the protocol comprises repeated cycles of enzymatic degradation and mechanical disintegration (Denef et al., 1980). Thyroid glands are dissected from connective tissue and cut into small pieces before incubation in a solution containing collagenase, DNAse and trypsin inhibitor followed by filtration and centrifugation at low speed in order to separate the follicles from large indigestible remnant tissues and single cells. Thus, the preparation is essentially free from fibroblast, endothelial cells and blood elements.

Selection of thyroid cancer cell lines

In paper III we wanted to preferentially study BRAF^{V600E} mutant cells and we choose ATC derived SW1736 and PTC derived BCPAP, both having the mutation and recommended as safe regarding origin and without contamination in the study of Schweepe et al (Schweppe et al., 2008). Studies on cell lines not harbouring BRAF^{V600E} mutation (Hth7 with mutation of *NRAS* Q61R) are being initiated but paper III only includes SW1736 and BCPAP as of the time of writing of this thesis.

2*D and 3D cell culture*

Most experiments in paper I were conducted on normal porcine thyrocytes cultured in Transwell™ bicameral insert chamber system suitable for monitoring transport across a tight monolayer. The model has previously been used in several studies in our laboratory as it is

better than any other *in vitro* systems as it mimics the natural route of iodide transport in the thyroid (Ericson and Nilsson, 1996; Nilsson and Ericson, 1994; Norden et al., 2007). Establishment of an epithelial barrier is absolutely necessary for proper measurement of active transport that can be distinguished from passive flux through leaky junctions. To ensure this, both transepithelial resistance (TER) and potential difference (PD) were measured during the entire experiments. This was particularly important when cells were treated with growth factors or drugs for prolonged time periods. For paper I we also adopted a 3D model in which thyrocytes were cultured in a collagen gel. Use of collagen type I as matrix to study cell behavior in a 3D matrix has a long history (Elsdale and Bard, 1972). Earlier studies also showed that embedding thyroid follicles in a collagen gel stabiles the correct polarity presumably through providing an interaction of the cell membrane with an extracellular component to which the cell can adhere (Chambard et al., 1981; Garbi et al., 1984). Notably, collagen gel embedded follicles were previously used as a model to investigate cell migration induced by peptide growth factors (Nilsson et al., 1995; Westermark et al., 1991). In paper I a modified version of the collagen gel culture in which follicles were allowed to reconstitute prior to experimental start provided a model in which functional dedifferentiation focusing on *pNIS* expression and iodination in response to chronic EGF stimulation was studied. In paper II this model was further employed to investigate involvement of MEK and PI3K pathways in EGF-induced cell migration. In paper III we used tumor cell lines also embedded in collagen and extended the single gel culture into invasion assay consisting of a double gel setup, we studied the effects of inhibitors.

Iodide transport and iodination

In paper I iodide transport and iodination was studied in 2D and 3D culture respectively. This was done by adding trace amount of $^{125}I^-$, only to the basal medium in Trasnwell™ chambers, and the measuring the accumulated activity either in the apical medium or trapped in follicles depending on type of experiment. In filter-cultured cells it is possible to differentiate between iodide transport through the epithelial cells including both the basolateral uptake mediated by NIS and apical efflux of iodide (Nilsson et al., 1990; Nilsson and Ericson, 1994) although this was not pursued in this thesis. To avoid confounding iodination in iodide transport studies, it is essential to inhibit TPO with methimazole (MMI). In collagen gel-cultured follicles it was not possible to distinguish $^{125}I^-$ uptake from unspecific binding of $^{125}I^-$, which required long rinsing to remove. However, in the absence of TPO inhibitor organification primarily representing iodinated TG is readily measured in the 3D model. As this was found to be

abolished by perchlorate, it is evident that the trapped activity depends on active transport by NIS and also that the cells formed tight follicles with a secluded lumen in which iodinated TG is stored. Conversely, 2D cultured cells on filter were not suitable for iodination as the radiotracer is rapidly diluted in the large apical medium volume away from the apical membrane where iodination normally take place (Ekholm, 1981; Ekholm and Bjorkman, 1997). Thus, both the models used in this thesis are complementary in experimental investigation of thyroid function.

NIS mRNA expression

NIS expression was analyzed by RT-qPCR in paper I. Primers used have previously been designed for to detect all transcripts present in porcine cells (Norden et al., 2007) but the reference gene was replaced by ubiquitin since the long term treatments radically affected other genes tested as for example 18s, GAPDH or beta actin . RNA was extracted from either 2D or 3D cultures by using RNeasy micro kit (Qiagen) and in both cases by direct lysis but an additional centrifugation step was included for 3D cultures to remove residual medium before the lysis. Reverse transcription was performed on the extracted RNA and qPCR was run using SYBR green method and relative expression was analyzed with ΔΔCt method (Livak and Schmittgen, 2001)

Analysis of phosphorylated ERK1/2 or AKT

Downstream signaling and protein analysis of phosphorylated ERK1/2 or AKT was performed by SDS PAGE and Western blot. Cells cultured in 2D were directly lysed in the presence of inhibitors of proteinases and phosphatases. In 3D cultures three gels were pooled and collagenase treated (100 U/ml) for 1 hour before the cells were pelleted and lysed. The transferring of the proteins to a membrane being probed for phosphorylated protein and after removing the first antibodies also reprobing it for total ERK1/2 or AKT for loading control.

Cell proliferation and survival

Cell proliferation was estimated by [^{3}H] thymidine incorporation in paper II and III. After incubation, excessive activity was removed by extensive washing before solubilization of the gel and liquid scintillation counting. For 3D-cultured cell lines this method was technically problematic with inconsistent results although morphological analysis revealed difference in cell density after treatment with inhibitors. Evaluation of tumor cell number in collagen gel was therefore limited to microscopy. Nucleus was counterstained with 4',6-diamidino-2-

phenylindole dihydrochloride (DAPI). When stained with DAPI nucleus with apoptotic cells will display a characteristic pattern of nuclear condensation and fragmentation which can be used to evaluate the viability of the cell. This is also important because the cells were treated for long time with drugs. Quantification of follicle number based on DAPI staining (paper II) was done in a standardized manner in which all follicles in four fields at 20X were counted.

Migration and invasion assays

In both paper II and III the migration and invasive behavior of normal follicular cells and thyroid cancer cells were investigated. In paper II this was based on reconstituted follicles and hence the starting point was cells in a highly differentiated state. Cell migration was evaluated by direct microscopy and quantified by counting the number of follicles with a typical pattern of radial protrusion of cells from the core epithelial lining. In paper III the migration capacity in human cancer cell lines was studied by scratch wounding at high cell density followed for 24 h of recovery. Scratch wounding is a classical *in vitro* method to estimate migration potential of cells, that lately also have been adapted to high-throughput screening in 384-well format (Yarrow et al., 2004). Since cell behavior can be modulated in presence of extracellular matrix we also wanted to study SW1736 and BCPAP when embedded in collagen. Migration of cells randomly dispersed in gels was impossible to evaluate so we developed the 3D model by embedding the cell-containing gel in another gel which made it possible to monitor and grade the amount of cells invading from the first to second gel over time by light microscopy. Invasion was documented by images taken at four opposite locations of the gel interface.

When following migration *in vitro* with the routine or invented methods used in this thesis, it is impossible to completely distinguish migration from potential proliferation which also could contribute to the covering of scratch wound or the increased number of cells in the outer second gel. Since results of scratch wounding were evaluated after 24 h this avoided a major contribution of proliferation. Invasion into the second gel was extended for longer time (3-6 days) hence proliferation likely influenced the number of cells.

Fluorescence microscopy in 3D cultures

Immunofluorescent labeling of 3-D cultured cells required optimization of protocols to reduce background staining. This included incubation overnight with blocking solution and primary antibody, prolonged incubation with secondary antibody, and extended time for washing. The intensity and distribution of TG immunoreactivity (cytoplasmic or lumen) were analyzed in

paper I and localization of ZO-1 was imaged in paper II to evaluate EGF and drug effects on thyroid function and epithelial phenotype respectively. In addition, F-actin labeled with phalloidin-FITC was analyzed in paper III to characterize cell shape changes of 3D-cultured cancer cell lines. Imaging of fluorescent signal in thick specimens and in which the objects of interest are dispersed in many focal planes is challenging. In an attempt to improve the evaluation, follicles were also cultured in a sandwich model seeded between two acellular gels. However, this method was rejected due to unwanted spontaneous spreading of cells forming a monolayer in the intervening space.

Results and Discussion

Switching from MAPK-dependent to MAPK-independent repression of the sodium-iodide symporter in 2D and 3D cultured normal thyroid cells (paper I)

The main findings in paper I were:

- Serum potentiates TSH-stimulated *NIS* expression and iodide transport in porcine thyroid epithelial cells 2D-cultured on a permeable filter.
- In this system, EGF down-regulates *NIS* expression in both a MEK-dependent and MEK-independent manner depending on the serum concentration.
- In thyroid cells embedded in collagen gel, serum is important for the viability and reconstitution of follicles but does not potentiate *NIS* expression or iodination.
- In reconstituted follicles EGF down-regulates NIS and prevents iodination entirely by a MEK-independent mechanism.
- Other investigated thyroid functions i.e. synthesis and turnover of TG and transepithelial electrolyte transport, are inhibited by activation of the MAPK signaling pathway.

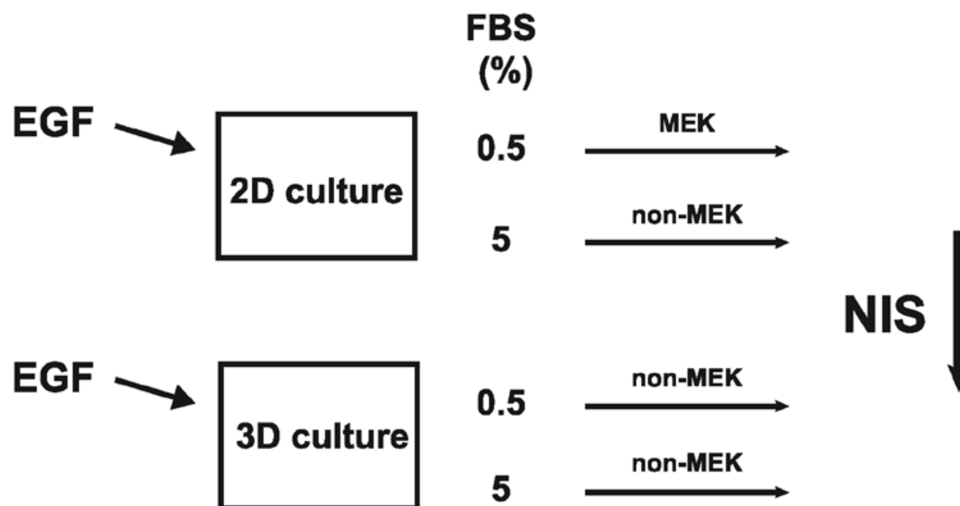

Fig.4. Interplay between FBS and MEK and Non-MEK dependent pathways leading to downregulation of NIS in 2D and 3D cultures of thyroid cells.

Refractoriness to radioiodine (iodine-131) in advanced thyroid cancer is primarily caused by loss of NIS expression (Schlumberger et al., 2007) and this has been found to be difficult to restore, which would be preferable since it would increase the possibilities to offer radiotherapy to patients with relapsing or widespread tumors. Interestingly, in a mouse model of $BRAF^{V600E}$-induced PTC it was found that when the oncogenic signal is shut down or MEK is inhibited, the expression of thyroid proteins TSH, TG and TPO was restored but NIS did not respond to the inhibitor as efficiently (Chakravarty et al., 2011). To further study MAPK involvement in down-regulation of *NIS* and iodide transportation we modeled this by stimulating normal thyroid cells cultured either on Transwell filters or in a collagen gel with EGF. In 2D culture, two different mechanisms were found. Firstly, in low serum conditions where the cells had a suboptimal TSH response in comparison to what was possible to achive in high serum conditions, EGF had a pronounced down-regulating effect on *NIS* expression and iodide transport, but over time the barrier function was lost which made it problematic to study iodide transport after long term treatment. In this low serum condition all parameters (apart from NIS-mediated transport also electrolyte transport and the barrier function) were readily restored in the presence of the MEK inhibitor U0126, suggesting a MEK-dependent mechanism responsible for EGF-induced thyroid dedifferentiation. When the serum level was raised to 5% during EGF-stimulation the MEK inhibitor still could prevent phosphorylation of ERK1/2 and also inhibit the loss of electrolyte transport, but interestingly *NIS* mRNA levels remained low indicating that in this condition the repression of *NIS* gene transcription is MEK-dependent only to a minor extent and a major MEK-independent mechanism is responsible. It should be mentioned that this shift was not due to reactivation of the MAPK signaling pathway, which is a common mechanism in tumor cells leading to acquisition of drug resistance to small molecule kinase inhibitors targeting this pathway (Alcala and Flaherty, 2012). In addition, poor recovery of NIS expression did not seem to be mediated by the PI3K pathway, which was found to contribute little if any to EGF responses in confluent primary cultured thyroid cells. So the findings were paradoxical with serum factor(s) on one hand promoting a differentiated state and high iodide transport capacity of the thyrocytes and on the other hand modifying the EGF response leading to persistent loss of *NIS* expression.

It has been shown that when thyroid cells are organized in a follicle-like structure *NIS* is expressed more (Bernier-Valentin et al., 2006). In addition, in breast epithelial cells 3D microenvironment also regulates gene expression (Bissell et al., 2003). So we decided to

culture the same thyroid cells as follicles embedded in a 3D matrix of collagen which potentially could give more insight in how serum, EGF and TSH modify the response and the effect of MEK inhibition. It was very interesting to find that even though serum was still important for the reconstitution of follicles early after plating serum concentration did not seem to affect function, that is the follicles responded equally well at both short and long term-stimulation with TSH regardless if the level of serum was kept high or lowered. Moreover, MEK inhibition was inefficient and could not rescue *NIS* expression or iodination. On the other hand, both synthesis and distribution of TG was reversed when U0126 was added. Hence, similar to the aforementioned mouse PTC model (Chakravarty et al., 2011), although both NIS and TG are affected by activation of the MAPK signaling pathway, the ability of small kinase inhibitors against MEK to reverse the phenotype differs with NIS being more refractory. Together, this suggests that a MEK independent mechanism more specifically repress the expression of *NIS*.

Dual contribution of MAPK and PI3K in epidermal growth factor-induced destabilization of thyroid follicular integrity and cell migration into extracellular matrix (paper II)

The main findings of paper II were:

- EGF-induced proliferation of thyroid cells cultured as reconstituted follicles in collagen gels involves both MAPK and PI3K signaling.
- In the same time EGF antagonizes TSH-stimulated enlargement of the follicle lumen, although in absence of kinase inhibitors the follicles remain intact.
- Radial cell migration induced by EGF is also dependent on MEK and PI3K, augmented by serum, and concomitant inhibition is needed for full inhibition of migration.
- Single treatment with MEK inhibitor only partly inhibited migration but fully restitutes the follicular structure.
- Single treatment with PI3K inhibitor adversely promotes EGF-induced cell migration and in addition provokes follicle disintegration.
- As revealed by ZO-1 staining, EGF treatment in the presence of PI3K inhibitor allows migrating cells to retain the epithelial phenotype with capacity to reform follicles.
- EGF exerts a cytoprotective effect that counteracts apoptosis resulting from PI3K inhibition.

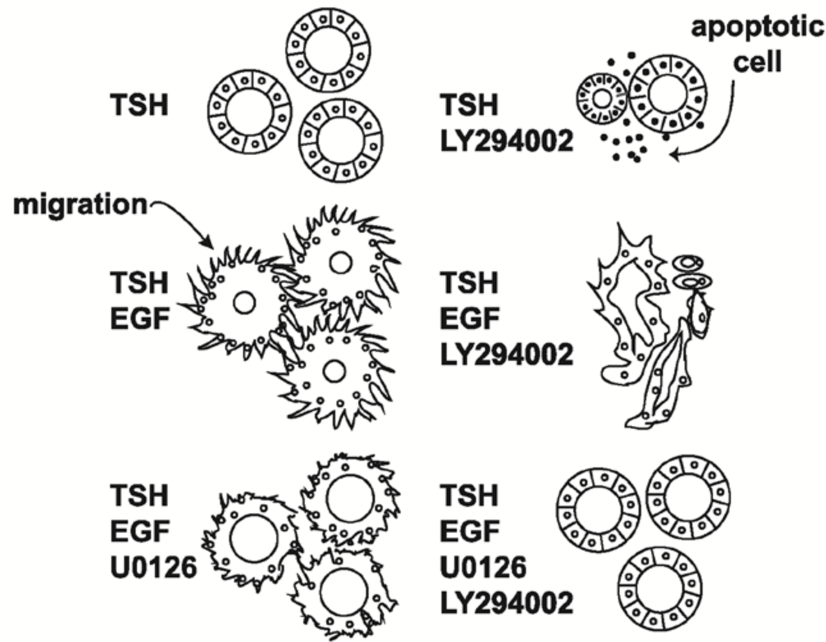

Fig.5. A representation of the morphological changes induced by various treatments used in this study.

In paper II we wanted to further investigate the collagen gel model focusing first on the possibility to pharmacologically block thyroid cell migration in a 3D context and, secondly, on possible mechanisms that may keep the integrity of the follicular epithelium during EGF-induced migration. Since PI3K signaling, in addition to the more well studied MAPK pathway has been suggested to contribute to thyroid cancer cell spreading (Burrows et al., 2013), we also wanted to include this pathway in the evaluation. First, we monitored cell proliferation based on [^{3}H]thymidine incorporation and found that this was increased by EGF regardless of serum content, although the effect was prominent in serum-starved cells. Inhibition of MEK or PI3K with U0126 and LY294002, respectively, reduced growth and when drugs were combined an additive effect was observed. We then analyzed the morphology of gel-cultured follicles treated with combinations of TSH, EGF, serum and inhibitors and also quantified the number of follicles with signs of cell migration. Radial migration was clearly induced by EGF and in addition there was a pronounced effect on the lumen structure as compared to TSH-stimulated follicles. The lumen structure was clearly restored in presence of U0126 regardless of serum but the inhibitory effect on cell migration was more obvious in low serum conditions. Thus, in follicles cultured in 5% FBS and stimulated with EGF there was a significant amount of ongoing migration when MEK was

inhibited from the start of experiment. Combined treatment with U0126 and LY924002 was needed to inhibit cell migration completely.

When cells were stimulated with EGF in the presence of a PI3K inhibitor, remarkable morphological changes were observed by direct microscopy, as if the entire follicular structure was abolished due to augmented cell migration. This pattern was especially pronounced at 5% FBS, although still obvious in reduced serum. However, immunolocalization of ZO-1 revealed that despite the chaotic growth pattern most cells were organized in follicles although with much distorted shapes and the lumens were still delimited by tight junctions with seemingly normal appearance. Interestingly, small aggregates of cells frequently found in cultures with combined EGF and LY294002 treatment expressed ZO-1 and had lumens. Counting them showed significantly increased numbers of lumen-containing structures only in this treatment group. It should be reminded that this was observed despite the fact that cell proliferation was reduced (by PI3K inhibition), indicating that new follicles were formed not because of increased cell number but rather reorganization of preexisting cells after breakdown of the original follicles. Hence, PI3K seems to be important for keeping the follicular structure intact. There is a considerable cross-talk between PI3K and MAPK signaling and PI3K can influence MAPK pathway and vice versa. Furthermore, depending on the situation PI3K can potentially even prolong the period of time when ERK is being phosphorylated (Aksamitiene et al., 2012). When added to TSH-stimulated cells LY294002 did not induce any migration, but morphologically the follicles appeared affected and DAPI staining revealed an increased number of cells with condensed or fragmented nuclei indicative of apoptosis. However, in the presence of EGF this pattern did not occur. Therefore, EGF supports cell survival that otherwise is threatened by repression of vital PI3K-dependent cellular functions.

Differential effects of MAPK pathway inhibitors on migration and invasiveness of BRAF^{V600E} mutant thyroid cancer cells in 2D and 3D culture (paper III)

The main findings of paper III were:

- Comparing two thyroid cancer cell lines of different tumor origin (BCPAP from PTC and SW1736 from ATC) BCPAP was more affected by PLX4720, a BRAF^{V600E}-specific inhibitor, and U0126 in all parameters evaluated.
- Rebound activation of MAPK/ERK1/2 pathway after PLX4720 treatment is evident in SW1736 but not in BCPAP although both harbor the BRAF^{V600E} mutation.
- Migration of SW1736 in 2D culture (wound assay) is largely resistant to both PLX4720 and U0126, although both inhibitors blocked cell proliferation.
- In 3D culture BCPAP cells are highly dependent on MAPK pathway for survival while SW1736 cells are much less sensitive.
- Single or dual inhibition of BRAF and MEK in 3D-grown SW1736 hamper cell migration but do not prevent matrix invasion during prolonged culture.

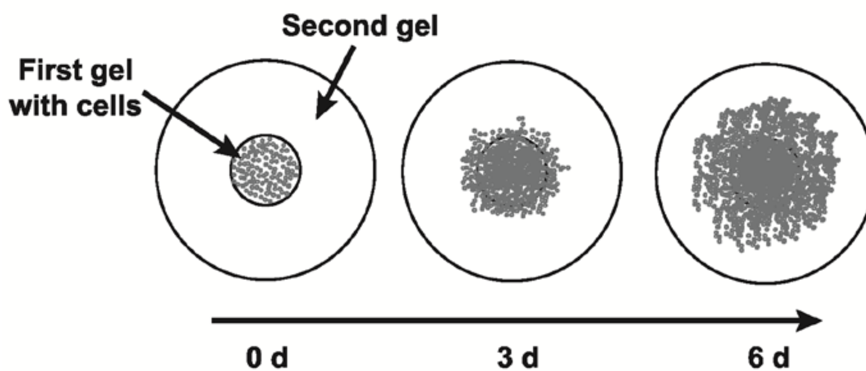

Fig.6. Overview of the 'gel-in-gel' method employed to culture thyroid cancer cells in this study.

Migration assays *in vitro* do not normally take microenvironment and extracellular matrix into consideration. In paper III we wanted to focus on the major oncogenic alteration in PTC, BRAF^{V600E} mutation, and its possible contribution to migration and invasion by setting up a 3D culture system and comparing this with findings in a conventional scratch wound assay in 2D culture. Two different cells lines were chosen, the PTC-derived BCPAP and ATC-derived SW1736. To evaluate the influence of MAPK signaling we selected one powerful MEK inhibitor, U0126, which is commonly used in experimental studies, and one BRAF^{V600E} inhibitor, PLX4720, which is the predecessor to PLX4032 or vemurafenib used in clinical

trials. Based on phosphorylation of ERK1/2, both cell lines were clearly inhibited by both substances administered one at the time but as previously reported for thyroid cancer cell lines (Montero-Conde et al., 2013) SW1736 showed a pronounced rebound activation of MEK in presence of PLX4720. However, rebound signaling was abolished with dual treatment, that is MEK remained silenced. Somewhat surprisingly, it was not necessary to prevent reactivation of MEK to sustainably inhibit tumor cell proliferation.

Next, we evaluated the potential effect of MAPK inhibition on the migration of tumor cells as monitored after scratch wounding of confluent 2D cultures. This showed major differences in drug response between the cell lines. First of all, BCPAP migrated more slowly than SW1736 and was unable to close the wound after 24 hours, which SW1736 did. BCPAP was also very sensitive to MEK or BRAF inhibition. SW1736 on the other hand was less affected and combined drug treatment did not produce a significant difference.

When embedded in collagen type I matrix cell proliferation was lowered in both cell lines as compared to 2D cultures. It should be mentioned that it was difficult to evaluate drug effects on growth after [^{3}H] thymidine labeling due to large variations within and between experiments. As an alternative method we stained F-actin, which made it easy to evaluate cell density in the entire gel volume. This showed that SW1736 increased in number between 24 and 72 hours whereas the amount of BCPAPs was fairly constant during the same time. In the presence of inhibitors, most notably U0126, the cell viability of BCPAP was markedly reduced with very few cells remaining after treatment for 48 hours. U0126 inhibited 3D growth of SW1736 but there were no evident signs of reduced survival. SW1736 multiplied in the presence of PLX4720, and the combined treatment with MEK inhibitor had no obvious additive effect.

Finally, we wanted to study drugs effects on matrix invasion of the cell lines. To this purpose a new model that allowed directed migration to be monitored embedding the cell-containing gel in a second gel of collagen. Cells entering the second, outer gel were monitored by direct microscopy for as long as 6 days without signs of side effects due to prolonged culture. In the absence of inhibitors, both cell lines migrated into the outer gel with a clear difference demonstrating that SW1736 has a much more invasive phenotype. Drug-treated BCPAP were unable to invade, which was expected by the finding most cells died under treatment. Also, the migration of SW1736 was much reduced. However, neither single nor dual treatment with PLX4720 and U0126 prevented matrix invasion completely, that is many tumor cells entered

the second gel after 6 days of treatment. Together, this suggests involvement of a MAPK-independent mechanism in migration of SW1736 and that targeting this pathway alone is insufficient to prevent tumor cell invasion.

Concluding Remarks

Regarding thyroid dedifferentiation:

- EGF-induced down-regulation of TG is MAPK dependent, whereas the negative effect on NIS expression occurs in two ways: the classical MAPK signaling and a non-MEK mechanism.
- Culture context, 2D or 3D, determines which mechanism predominates and whether MAPK inhibitors can restitute NIS expression or not.
- The findings offer a new explanation for loss of iodide uptake in response to receptor tyrosine kinase activation.

Regarding thyroid cell migration:

- EGF-induced cell migration in 3D-cultured thyroid follicles is concerted by MAPK and PI3K-mediated signals, and both pathways must be blocked to prevent matrix invasion.
- PI3K-regulated mechanism(s) supervise maintenance of follicular structure and integrity of the epithelium, ultimately supporting thyroid cell survival. Inhibition of PI3K under activation of the MAPK pathway provokes follicle disintegration and accelerated cell migration.
- This motivates a cautionary note of a potentially disadvantageous effect of PI3K inhibitors unless MAPK signaling is simultaneously blocked.

Regarding thyroid cancer cell invasion:

- $BRAF^{V600E}$ mutant cell lines derived from different types of thyroid cancer possess different sensitivity to BRAF and MEK inhibitors regarding tumor cell migration and invasion.
- Rebound activation of MEK after BRAF inhibition does not necessarily correlate with gain of tumor cell growth or altered tumor cell motility.
- Dual inhibition of BRAF and MEK do not prevent matrix invasion of 3D-cultured thyroid tumor cells derived from anaplastic cancer. Such treatment may select tumor subclones with a higher invasive potential.

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