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Identification of novel protein kinases involved in the proliferation of thyroid cancer cells

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Identification of novel protein kinases involved in the proliferation of thyroid cancer cells

Doctoral dissertation

LIST OF PUBLICATIONS

This dissertation is based on the following manuscripts. The first one covers the main body of the dissertation. The others are appended at the end.

- Castellone MD*, **Cantisani MC***, et al., Identification of novel kinases involved in the proliferation of thyroid cancer cells. **Equally contributing authors*. Manuscript submitted (main body of the dissertation).
- Castellone MD*, **Cantisani MC***, et al., Sonic Hedgehog pathway is upregulated in thyroid cancer. **Equally contributing authors.* Manuscript in preparation (appended at the end of the dissertation).
- Laatikainen LE, Castellone MD, Hebrant A, Hoste C, **Cantisani MC**, Laurila JP, Salvatore G, Salerno P, Basolo F, Nasman J, Dumont JE, Santoro M, Laukkanen MO. Extracellular superoxide dismutase is a thyroid differentiation marker down-regulated in cancer. Endocr Relat Cancer. 2010 Aug 16;17(3):785-96.

TABLE OF CONTENTS

ABBREVIATIONS

ATC	anaplastic thyroid carcinoma
ATP	adenosine triphosphate
CML	chronic myeloid leukemia
CREBL2	cAMP responsive element binding protein 3-like 2
CTNNB1	catenin (cadherin-associated protein) beta 1
DMEM	Dulbecco's modified Eagle's medium
EFN	Eph-receptor ligand (ephrin)
EGFR	epidermal growth factor receptor
EPH	erythropoietin producing-hepatocellular carcinoma
EPHRIN	Eph-receptor ligand
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
FDA	food and drug administration
FGFR	fibroblast growth factor receptor
FMTC	familial medullary thyroid carcinoma
FNMTC	familial non medullary thyroid carcinoma
FTC	follicular thyroid carcinoma
GDNF	glial cell line-derived neurotrophic factor
GFP	green fluorescent protein
GFRα	GDNF-family receptor α
GIST	gastro-intestinal stromal tumor
HSPG	heparin-sulfate proteoglycans
HUVEC	human umbilical vein endothelial cell
LMW-PTP	low-molecular weight phosphotyrosine phosphatase
LOH	loss-of-heterozygosity
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase

MEN2	multiple endocrine neoplasia type 2 syndrome
MSP	major sperm protein
MTC	medullary thyroid carcinoma
NGFR	nerve growth factor receptor
NTRK1	neurotrophic receptor-tyrosine kinase 1
PDC	poorly differentiated carcinoma
PDGFR	platelet-derived growth factor receptor
PDZ	postsynaptic density 95/disc-large/zona occludens
PI3K	phosphatidylinositol 3-kinase
PIK3CA	phosphatidylinositol 3-kinase p110 coding gene
PPAR	peroxisome proliferator-activated receptor
PPFP	PAX8-PPARγ fusion protein
PTB	protein tyrosine-binding
PTC	papillary thyroid carcinoma
PTK	protein tyrosine kinase
RET	Ret fused gene
RISC	RNA-induced silencing complex
RTK	receptor tyrosine kinase
SAM	sterile α motif
SH2	src homology 2
shRNA	short-hairpin RNA
siRNA	small interfering RNA
TFG	TRK-fused gene
TPM3	tropomyosin 3
TPR	translocated promoter region
TSHR	thyroid stimulating hormone receptor
TTF1	thyroid transcription factor 1
TTF2	thyroid transcription factor 2
VEGFR	vascular endothelial growth factor receptor

1. ABSTRACT

This dissertation focuses on the study of different signaling mechanisms regulating thyroid carcinogenesis, with the aim of identifying new genes involved in thyroid tumors, thus potential targets for new anti-cancer therapeutics. Appended manuscripts **II** and **III** focus on the analysis of the role exerted by SOD3 (Superoxide dismutase 3) and components of the Sonic/Hedgehog signaling pathway in thyroid carcinomas. The main body of this dissertion (manuscript **I**), instead, describes a knock-down screening performed in the TPC1 thyroid cancer cell line with a library of siRNA targeting the entire complement of human protein kinases (kinome) and kinase-related proteins. Thyroid cancer is primarily associated to the oncogenic conversion of protein kinases (BRAF, RET, NTRK1, AKT). Thus, the aim of this screen was to identify novel protein kinases required to sustain viability of thyroid carcinoma cells and therefore involved in thyroid carcinogenesis. Through this screening, we identified 21 “down hits”, e.g. kinases whose knock-down reduced by 30% or more TPC1 cell viability. Most of these kinases were essential not only for the viability of TPC1 but also other thyroid cancer cell lines with different genetic backgrounds (RAS or BRAF mutations) but not normal thyroid cells. These kinases included components of several signaling pathways. In particular, we identified members of the EPH (ephrin receptors) family, namely EPHA2, EPHA4 and EPHB2, as overexpressed and functionally active in various thyroid cancer cell lines.

These three EPH were also upregulated in human thyroid tumor specimens. Finally, functional assays proved that the expression of EPHA2, EPHA4 and EPHB2 is essential not only for thyroid cancer cell growth but also motility and invasiveness. In conclusion, our study strongly suggests that EPHA2, EPHA4 and EPHB2 activation plays an important role in human cancers derived from thyroid follicular epithelium.

2. BACKGROUND

2.1 Thyroid tumors.

In mammals, the thyroid gland is located on the anterior surface of the trachea at the base of the neck. It is composed of two different lobes, each formed by two cell types. Follicular cells are epithelial cells organized in follicles, spherical structures serving as storage and controlling release of L-tri-iodothyronine (T3) and L-thyroxine (T4) thyroid hormones, under the control of the hypothalamic-pituitary axis. Parafollicular cells or C-cells, are scattered in the interfollicular space, mostly in a parafollicular position; they originate from the neural crest and are responsible for the production of the calcium-regulating hormone calcitonin. Thyroid neoplasms can derive from follicular cells or C-cells. Follicular cell-derived lesions are broadly subdivided in benign and malignant (Mazzaferri, 1993).

Benign thyroid lesions are typically solitary adenomas (Kondo *et al.,* 2006). They can be clinically silent or hyperfunctioning (referred to as a toxic thyroid adenoma), thereby causing hyperthyroidism with increased levels of thyroid hormones. Gain-of-function mutations of TSHR (~80%) and GNAS1, encoding GSα, (~25%) are the most common genetic lesion in functional thyroid adenomas, whereas these genetic lesions did not occur in thyroid malignancies (Parma *et al*., 1995).

Malignant thyroid lesions are the most common cancers of endocrine organs and represent approximately 1% of newly diagnosed cancer cases (Hundahl *et al*., 1998; Gimm, 2001), with an incidence rate stably increasing over the past few decades (Jemal *et al*., 2010). Cancers deriving from follicular cells represent more than 95% of thyroid cancers whereas only 3-5% of them arise from C-cells. On the basis of histological and clinical parameters, malignant follicular-derived lesions are classified into well differentiated carcinomas (WDC), poorly differentiated carcinomas (PDC), and anaplastic carcinomas (ATC, 2%) (Tallini, 2002; DeLellis and Williams, 2004; DeLellis, 2006; Kondo *et al*., 2006).

At least in some cases, thyroid carcinogenesis may be explained by a multi-step model (Fig. 1). According to this model, well-differentiated tumors (PTC and FTC) occur after early initiating events such as RET/PTC rearrangements and BRAF mutations or RAS mutations and PAX8-PPARγ rearrangement (Fusco *et al*., 2002), whereas adjunctive mutations of genes like p53, CTNNB1 and PI3KCA lead to ATC (García-Rostán *et al*., 2001; García-Rostán *et al*., 2003; García-Rostán *et al*., 2005). Cyclin D1 overexpression or p27 downregulation are implicated particularly in aggressive WDC subtypes (Tallini *et al*., 1999; Tallini, 2002; Kondo *et al*., 2006).

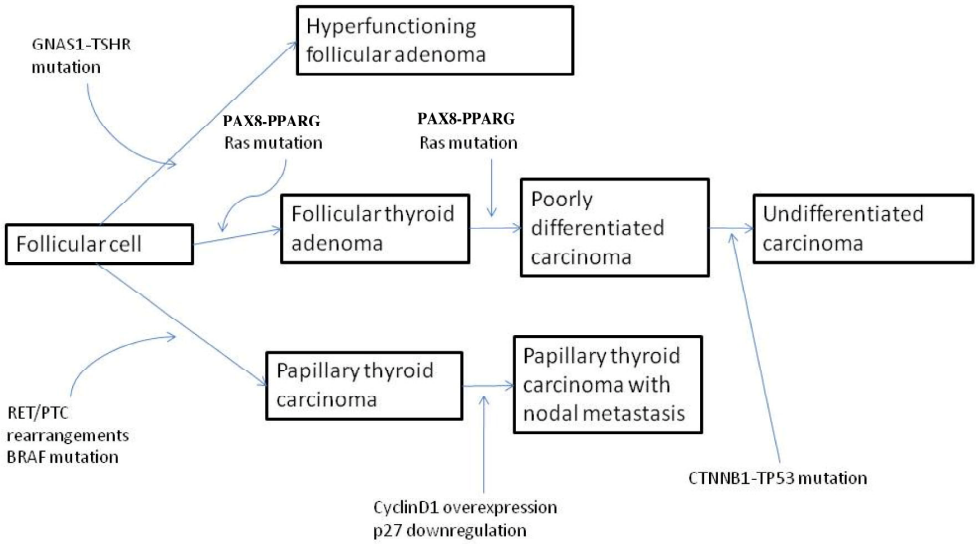

Figure 1: Model of thyroid multi-step carcinogenesis.

Recently, Todaro *et al*., showed that in thyroid cancer, tumorigenic activity is confined to a small subpopulation of stem-like cells, which are sustained by constitutive activation of cMet and Akt. This stem cell population is increased in ATC comparing to WDC. Moreover, a particular combination of genetic lesions, as BRAF and p53 mutations, may expand this compartment and favour the formation of more aggressive cancer types (Todaro *et al*., 2010).

- *WDC*

Well-differentiated carcinomas represent more than 90% of thyroid cancer cases and are divided in papillary (PTC, 85%) and follicular (FTC, 5-10%) thyroid carcinoma (Pacini *et al.,* 2006). PTC is characterized by a branching (papillary) architecture and peculiar nuclear features, but several variants are known (DeLellis, 2006), as tall-cell variant that display more aggressive features (Sherman, 2003; Leboulleux *et al.,* 2005; Adeniran *et al.,* 2006; Elisei et al., 2008a; Romei *et al.,* 2008). PTC is more frequent in women than men and affects patients 20-50 years old. PTC can also occur in childhood as consequence of accidental or therapeutic radiation exposure. PTC has an indolent behavior, tendency to form metastasis to local lymph nodes and a survival rate greater than 90% (Schlumberger, 1998; Sherman, 2003; DeLellis, 2006). Sometimes the disease may show an aggressive behavior and lose the ability to concentrate radioiodine.

FTC is characterized by follicular cell differentiation in the absence of the diagnostic nuclear features of PTC. It mostly affects 40-60 years old patients with a female to male ratio of 3-4:1 (DeLellis, 2006). Despite a higher frequency of distant haematogenous metastasis compared to PTC, most FTC patients can also be cured, with good long-term survival rates (Schlumberger, 1998; Sherman, 2003; Durante *et al.,* 2006).

- *ATC*

ATC is a tumor composed, entirely or partially, of undifferentiated cells (DeLellis, 2006), thus usually with a poor prognosis. According to a multi-step model discussed in figure 1, ATC may derive from a pre-existing well-differentiated carcinoma. This is suggested by the coincidental detection of WDC tissue in more than 25% of ATC patients (Pasieka, 2003; Ordonez *et al.,* 2004). ATC disseminates both to regional lymph nodes and to distant sites (Pasieka, 2003; Ordonez *et al.,* 2004).

- *PDC*

PDC represents 5% of thyroid cancers and it is defined as neoplasm of follicular origin that shows loss of structural and functional differentiation. Characteristically, these lesions show widely infiltrative growth, necrosis, vascular invasion and numerous mitotic figures (DeLellis, 2006; Pulcrano *et al.,* 2007; Volante *et al.,* 2007).

- *MTC*

Medullary thyroid carcinoma (MTC) is a rare malignant tumor that arises from C-cells (Elisei *et al.,* 2007; Elisei *et al.,* 2008b; Schlumberger *et al*., 2008). MTC comprises sporadic and autosomal dominantly inherited familial cases (Marx, 2005).

Familial MTC (FMTC) can present as isolated FMTC or associated to pheochromocytoma, parathyroid adenoma and other tumors in the context of MEN2 (*M*ultiple *E*ndocrine *N*eoplasia type 2) syndromes A and B (MEN2A, MEN2B) (Leboulleux *et al*., 2004; DeLellis, 2006).

2.1.1 Genetic lesions in thyroid tumors.

Familial occurrence of WDC has been described (Malchoff and Malchoff 2006; Sturgeon *et al*., 2005; Capezzone *et al*., 2008). Indeed, variants of TTF1 (NKX2-1) and TTF2 (FOXE1) thyroid transcription factor genes have been described in association with familial WDC (Gudmundsson *et al.,* 2009). Moreover, FNMTC (*F*amilial *n*on *M*edullary *T*hyroid *C*arcinoma) may occur in association with hereditary cancer syndromes, such as FAP (*F*amilial *A*denomatous *P*olyposis), Cowden disease, Carney complex and Werner syndrome. However, such familial clustering (syndromic FNMTC) accounts only for a small fraction of WDC cases (Malchoff and Malchoff 2006; Sturgeon *et al*., 2005; Capezzone *et al*., 2008).

Thyroid neoplasms are related to several risk factors, including reduced iodine uptake, lymphocytic thyroiditis and hormonal factors (estrogens). Recently, Antico-Arciuch and co-workers revealed a role for estrogens using a transgenic model of thyroid carcinogenesis (Antico-Arciuch *et al*., 2010). Radiation exposure is an important exogenous risk factor able to cause thyroid carcinoma.

Indeed, after the Chernobyl nuclear disaster, PTC frequency markedly increased in children exposed to radiations (Williams, 2002; Ciampi and Nikiforov, 2007). It is still unclear whether this particular occurrence in children was because the thyroid is most susceptible to radiation-induced damage in childhood, because children were exposed to contaminated milk or both. (Williams *et al*., 2002).

Ionizing-radiations associated PTCs have a high prevalence of RET/PTC rearrangements (see also below) and recently this genetic lesion has been mechanistically linked to radiation exposure. RET and H4 genes map in fragile chromosomal (chr. 10) sites and therefore can undergo DNA double strand breaks upon exposure to ionizing radiation. Moreover, in the interphase chromatin of thyroid cells, H4 and RET genes frequently overlap. This spatial proximity facilitates genetic recombination, giving rise to the H4-RET (RET/PTC1) oncoprotein (Nikiforova *et al*., 2000). Propensity of thyrocytes to increase DNA end-joining activity upon radiation-induced DNA damage may further allow RET/PTC chromosomal rearrangements to occur (Yang *et al*., 1997; Volpato *et al*., 2008; Gandhi *et al*., 2010).

The genetic lesions associated to thyroid carcinomas can affect either proto-oncogenes (gain-of-function mutations) or tumor suppressor genes (loss-of-function mutations). These lesions are listed in Table 1 and described in the following sections.

Tumor Type	*Cell Type*	*Molecular Lesion*
PTC	Follicular Cell	• RET/PTC rearrangements • NTRK1 rearrangements • BRAF point mutation
FTC	Follicular Cell	• PAX8/PPARγ rearrangement • RAS point mutation
ATC	Follicular Cell	• BRAF or RAS point mutation • p53 point mutation • CTNNB1 mutation • PI3KCA/AKT mutation
MTC	Parafollicular Cell	• RET point mutation

Table 1: Genetic defects in thyroid tumors.

- *Genetic alterations in PTC*

About 70% of PTC cases are associated to two types of genetic lesions: i) chromosomal rearrangements involving RET (*RE*arranged during *T*ransfection) and NTRK1 (*N*eurotrophic *R*eceptor-*T*yrosine *K*inase 1) proto-oncogenes and ii) point mutations in BRAF and RAS genes (Santoro *et al*., 1992; García-Rostán *et al*., 2003; Kimura *et al*., 2003; Soares *et al*., 2003; Nikiforova *et al*., 2003a; Nikiforova *et al*., 2003b; Frattini *et al*., 2004; Elisei *et al*., 2008a) (Table 1).

The most common events are those affecting RET and BRAF. These genes encode proteins that signal along the ERK (*E*xtracellular signal-*R*egulated *K*inase) pathway and the genetic alterations targeting them result in constitutive ERK1/2 pathway activation. A schematic representation of ERK pathway is illustrated in figure 2.

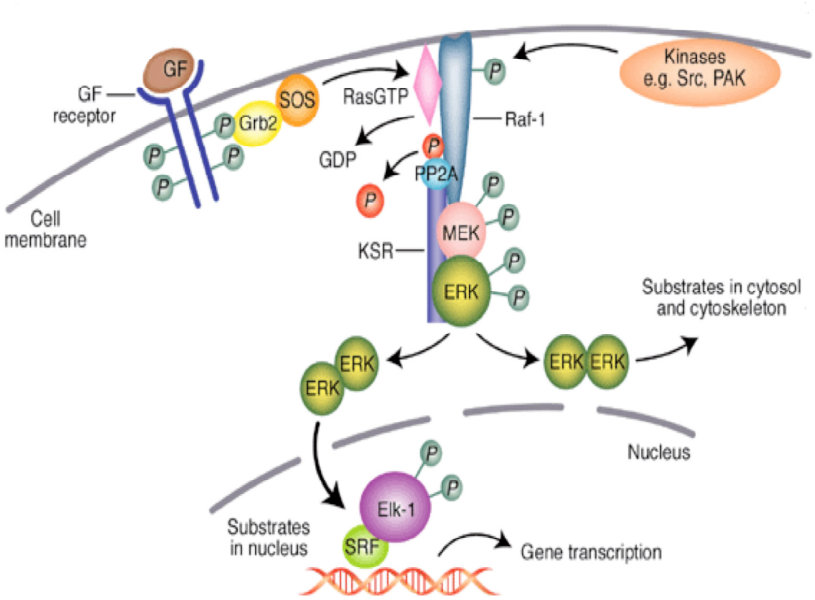

Figure 2: ERK signaling pathway.

After binding of a growth factor (GF) to its cognate TK receptor, a series of events including activation of RAS, RAF and MEK culminate in the activation of ERK kinases. Once in the nucleus, ERK activate several transcription factors.

- *RET*

The RET proto-oncogene is located on chromosome 10q11.2 and encodes a transmembrane receptor tyrosine kinase (RTK) with four cadherin-related motifs in the extracellular domain (Santoro *et al*., 2004). RET is normally expressed in the developing central and peripheral nervous system and is an essential component of a signaling pathway required for renal organogenesis and enteric neurogenesis. RET is normally expressed at high levels in C-cells, but not in follicular cells (Santoro *et al*., 2006). *G*lial cell line-*d*erived *n*eurotrophic *f*actor (GDNF)-family ligands (Manié *et al*., 2001; Ciampi and Nikiforov 2007) and GDNF-*f*amily *r*eceptor α (GFRα) bind the extracellular domain of RET inducing its dimerization. This active form of RET autophosphorylates specific tyrosine residues within its intracellular domain, which function as binding sites for signaling molecules containing phosphotyrosine-binding motifs (SH2 or PTB), thereby activating several signaling pathways (Santoro *et al*., 2004; Santoro *et al*., 2006). Through phosphorylated Y1062, RET binds SHC and FRS2, which recruit Grb2-SOS complexes leading to the activation of the RAS-RAF-ERK cascade (Asai *et al*., 1996; Melillo *et al*., 2001). Through pY1062, RET is also able to activate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Segouffin-Cariou and Billaud, 2000; Pelicci *et al*., 2002).

Gain-of-function mutations of RET are involved in sporadic MTC and FMTC, including MEN2A and MEN2B. Conversely, PTCs are characterized by chimeric oncoproteins, named RET/PTC, originating from the in-frame fusion of the RET encoding tyrosine kinase domain and a 5'-terminal of different partner genes (Tallini, 2002).

RET/PTC lacks RET signal peptide and transmembrane domains but retains the kinase domain and most of the autophosphorylation sites, thereby allowing downstream signaling (Ciampi and Nikiforov, 2007). By providing an active transcriptional promoter, RET/PTC rearrangements enable thyroid expression of the chimeric RET/PTC oncoproteins (Fusco *et al*., 1987; Grieco *et al*., 1990).

To date, more than 10 RET/PTC rearrangements have been described; the most frequent are RET/PTC1, which involves RET and H4 genes (Fusco *et al*., 1987; Grieco *et al*., 1990; Nikiforova *et al*., 2000) and RET/PTC3, between RET and RFG (*R*et-*F*used *G*ene) (also named NCOA4, ELE1 or ARA70) (Santoro *et al*., 1994; Borganzone *et al*., 1994) (Fig. 3). These oncoproteins induce transformation and de-differentiation of cultured thyroid cells (Santoro *et al*., 1993; De Vita *et al*., 1998), and thyroid-targeted expression of RET/PTC1 or RET/PTC3 induces thyroid neoplasms in mice (Santoro *et al*., 1996). RET/PTC rearrangements are found in 20-40% of PTC, with higher prevalence in classic form rather than in follicular variant (Tallini, 2002). Their prevalence is significantly higher in young age patients and in patients with a history of accidental or therapeutic radiation exposure.

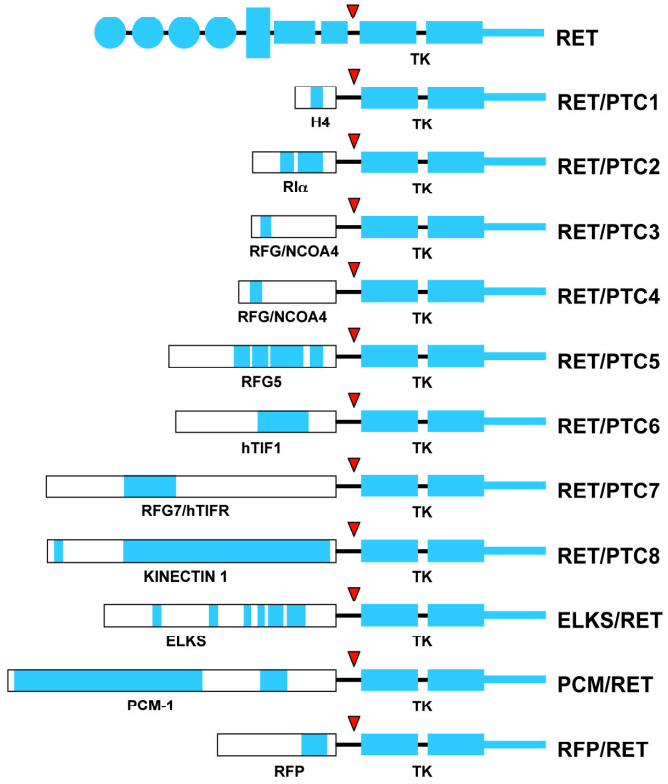

Figure 3: Most common RET/PTC rearrangements in papillary thyroid cancer. Red arrow: breakpoint.

RET/PTC1 is more frequently associated with classic PTC and with the diffuse sclerosing variant PTC; conversely, RET/PTC3 is more common in the solid variant and in PTC associated to ionizing radiation exposure (Thomas *et al*., 1999). Moreover, at a variance from BRAF (see below), RET/PTC it is not a negative prognostic factor for PTC (Elisei *et al*., 2008a). The high frequency of RET/PTC rearrangements in subclinical microcarcinomas is also consistent with the hypothesis that RET/PTC rearrangements are early events in thyroid tumorigenesis (Fusco *et al*., 2002).

On the other hand, the low prevalence of expression of RET/PTC oncoproteins in PDC and ATC suggests that they do not play a prominent role in thyroid tumor progression (Santoro *et al*., 1992; Tallini *et al*., 2001). RET/PTC signals along the ERK pathway (Knauf *et al*., 2003, Melillo *et al*., 2005, Mitsutake *et al*., 2005), but other pathways, particularly the PI3K/AKT one, may contribute to its biological effects as well (Pelicci *et al*., 2002; Miyagi *et al*., 2004; Jung *et al*., 2005, De Falco *et al*., 2005).

- *NTRK1*

Similar to RET, NTRK1, the high affinity receptor of NGF (*N*erve *G*rowth *F*actor), undergoes oncogenic activation by chromosomal rearrangements in PTC (Greco *et al*., 2004). At least three partner genes (TPR, TPM3 and TFG) are involved in these rearrangements (Greco *et al*, 1993; Greco *et al*., 1995; Greco *et al*., 1997). NTRK1 rearrangements appear less prevalent than RET/PTC ones (Greco *et al*., 2010) (Fig. 4).

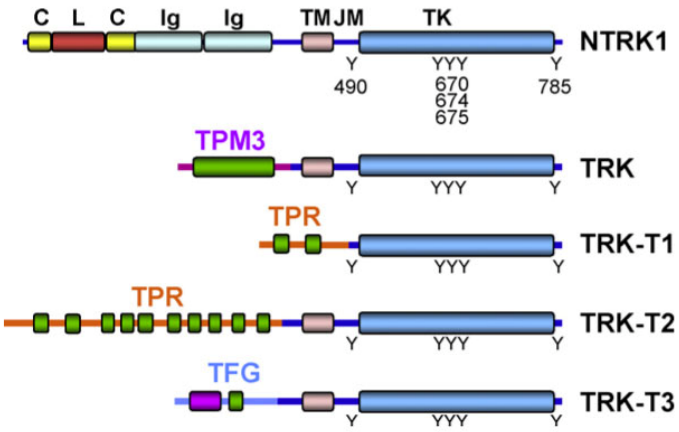

Figure 4: Most common TRK rearrangements in papillary thyroid cancer (Image adapted from Greco *et al.*, 2010).

C: cystein-rich domain; L: leucine riche domain; Ig: immunoglobulin like domain; TM: transmembrane domain; JM; juxtamembrane domain. Autophosphorylation (Y) sites are also shown.

- *BRAF*

Together with ARAF and CRAF, the proto-oncogene BRAF (7q24) belongs to the RAF serine-threonine kinase family that transduce regulatory signals through intracellular effectors of the MAPK/ERK pathway (Fig. 2). Among the RAF family members, BRAF has the highest basal kinase activity and is the most potent activator of the MAPK pathway (Wellbrock *et al.*, 2004). Activating point mutations within the kinase domain of BRAF have been found in several tumor types, including melanoma and colorectal cancer.

The most common BRAF mutation in human cancer, including PTC (Xing *et al*., 2007; Xing *et al*., 2010), is a transversion from a thymine to adenine at nucleotide 1799 (T1799A), resulting in a substitution of glutamic acid for a valine at residue 600 (V600E) of the protein. Follicular variant PTC shows more often the K601E mutation in BRAF. The in-frame VK600-1E deletion (BRAF $^{VK600\text{-}1e}$) has been identified in a solid variant of PTC (Xing, 2007). V600E and most other mutations within the BRAF kinase domain target either the activation loop (V600) or less frequently the ATP binding site (P loop). By disrupting the interactions between the activation loop and the P loop, that hold the kinase in an inactive conformation, these mutations cause BRAF constitutive activation (Wan *et al*., 2004). BRAF mutation are found in 29-69% of early stage of PTC (Ugolini *et al*., 2007) but not in FTC, and up to 13% of PDC and 35% of ATC (Nikiforova *et al*., 2003b). BRAF mutations are highly prevalent in classic and tall-cell variant PTCs, but similar to RET/PTC rearrangements, they are rarely found in the follicular variant PTC (Xing, 2007). Thyroid-specific BRAFV600E-transgenic mice develop tumors that quite closely recapitulate the features of human PTC. These transgenic PTCs show aggressive behavior and progress to PDC (Knauf *et al*., 2005). This suggests the involvement of BRAF mutation not only in PTC initiation but also in progression to PDC and ATC. Indeed, in human patients BRAF mutations correlate with aggressive tumor behavior, tumor recurrence, decreased radioiodine concentration ability and decreased survival (Namba *et al*., 2003; Kimura *et al*., 2003; Xing *et al*., 2007; Riesco-Eizaguirre *et al*., 2006; Lupi *et al*., 2007; Elisei *et al*., 2008a; Xing, 2010).

For these reasons, BRAF and the downstream MEK kinase can be considered as promising molecular targets for thyroid cancer treatment, in particular aggressive PTC variants as well as ATC (Santoro *et al*., 2006; Schlumberger *et al*., 2009; Sherman, 2009).

- *RAS*

RAS family members, namely HRAS, NRAS, KRAS, are small-GTPases activating MAPK pathway (Fig. 2), frequently activated in human cancers. RAS point mutations in codons 12, 13 and 61 are found in follicular variant PTC (Zhu *et al*., 2003; De Lellis 2006, Kondo *et al*., 2006) and in PDC and ATC cases (Table 1). These evidences indicate that RAS play a role in tumor progression (García-Rostán *et al*., 2003; Volante *et al*., 2009).

- *Genetic alterations in FTC*

FTC may develop through at least two different pathways, involving either RAS or PPARG (*P*eroxisome *P*roliferator-*A*ctivated *R*eceptor) genes (Table 1) (Nikiforova *et al*., 2003b). PPARG is a member of the steroid nuclear-hormone-receptor superfamily that forms heterodimers with retinoid X receptor. Almost 30% of FTC cases present a PAX8-PPARγ *f*usion *p*rotein (PPFP), due a balanced translocation between thyroid-specific PAX8 trnascription factor and the reading frame of the PPARγ gene (Eberhardt *et al*., 2010) (Fig. 5).

This event results in the fusion of the region encoding the DNA binding domains of PAX8 to the region encoding domains A-F of PPARγ. The resulting fusion oncoprotein acts as dominant-negative PPARγ (Kroll *et al*., 2000; Castro *et al*., 2006). Recently Lui and co-workers have identified a new rearrangement involving CREB3L2 and PPARγ genes implicated in the pathogenesis of FTC (Lui *et al*., 2008). Point mutations or gene amplification of PI3KCA have been reported in few FTC cases as well (García-Rostán *et al*., 2005; Liu *et al*., 2008). Moreover, increased incidence of FTC has been observed in the context of Cowden disease, caused by PTEN mutation (Liaw *et al*., 1997).

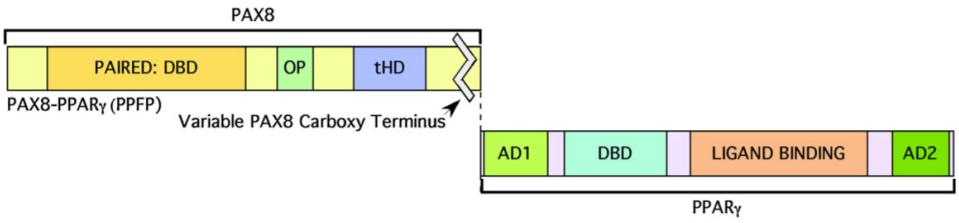

Figure 5: PPARG rearrangement in follicular thyroid cancer (Image modified from Eberardt *et al*., 2010).

DBD: DNA binding domain; OP: octapeptide motif; HD: homeobox domain; AD: activation domain.

- *Genetic alterations in PDC and ATC*

PDC and ATC share genetic lesions with WDC, consistent with the hypothesis of a multi-step model of thyroid carcinogenesis discussed below (Tallini, 2002; Nikiforov, 2004). They are charactherized by point mutations in RAS (García-Rostán *et al*., 2003; Volante *et al*., 2009), amplification or point mutation in PI3KCA (García-Rostán *et al*., 2005; Liu *et al*., 2008). Moreover, approximately 70% of ATC and a significant fraction of PDC show point mutations of TP53 (Nikiforov, 2004; Kondo *et al*., 2006). Up-regulation of negative p53 regulators or of proteins fostering p53 degradation can also negatively affect p53 function in ATC (Salvatore *et al*., 2007). About 30% of ATC and PDC harbor the V600E BRAF mutation, particularly those samples with morphological evidence of pre-existing PTC (Nikiforova *et al*., 2003; Soares *et al*., 2004; Begum *et al*., 2004). Another gene whose mutation has been associated to ATC is β-catenin (CTNNB1) (García-Rostán *et al*., 2001). This cytoplasmic protein, encoded by the CTNNB1 gene, plays an important role in E-cadherin-mediated cell-cell adhesion and it is also an important intermediate in the wingless (Wnt) signaling pathway. Point mutations in exon 3 of CTNNB1 have been reported in PDC and more frequently in ATC, but not in WDC (García-Rostán *et al*., 2001; Miyake *et al*., 2001), suggesting that they might play a direct role in the dedifferentiation and progression to ATC.

- *Genetic alterations in MTC*

As previously mentioned, MTC can occur either sporadically or as FMTC in the context of autosomal dominant MEN 2 syndromes (MEN 2A, MEN 2B and FMTC) (Manié *et al.*, 2001; Marx, 2005; de Groot *et al*., 2006). RET point mutation is so far the only genetic lesion consistenly associated to MTC (Fig. 6) (Elisei *et al*., 2007; Elisei *et al*., 2008b; Schlumberger *et al.,* 2008). In virtually all MEN2A cases, mutations target extracellular cysteine residues in RET. In more than 90% of the cases, MEN2B is caused by the Met918Thr intracellular substitution in the P+1 loop of the kinase. FMTC is caused by mutations in the RET extracellular or kinase domains. Sporadic MTC have somatic RET mutation, and up to 50% of the cases harbor the Met918Thr in RET (Cote and Gagel 2003; Leboulleux *et al*., 2004; De Groot *et al*., 2006) (Fig.6). Accordingly, RET kinase has emerged as a promising molecular target for treatment of MTC (Santoro *et al*., 2006).

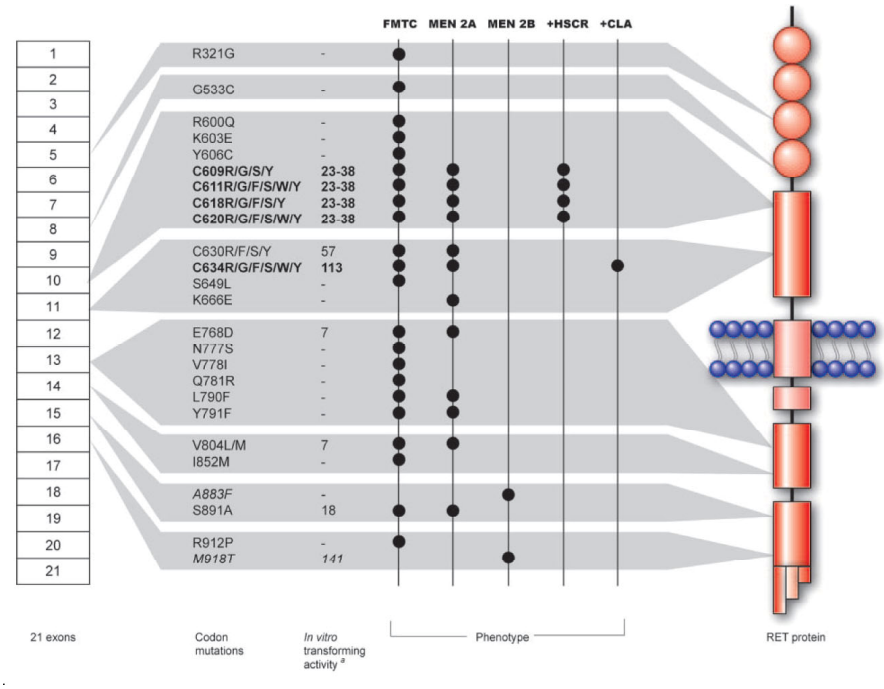

Figure 6: RET mutations in MTC (Image adapted From De Groot *et al*., 2006).

2.2 Loss-of-function genetic screening based on RNA interference.

RNA interference (iRNA) is a natural occurring mechanism controlling gene expression at a post-trascriptional level first identified in *C. elegans* in 1998 (Fire *et al.*, 1998) (Fig. 7).

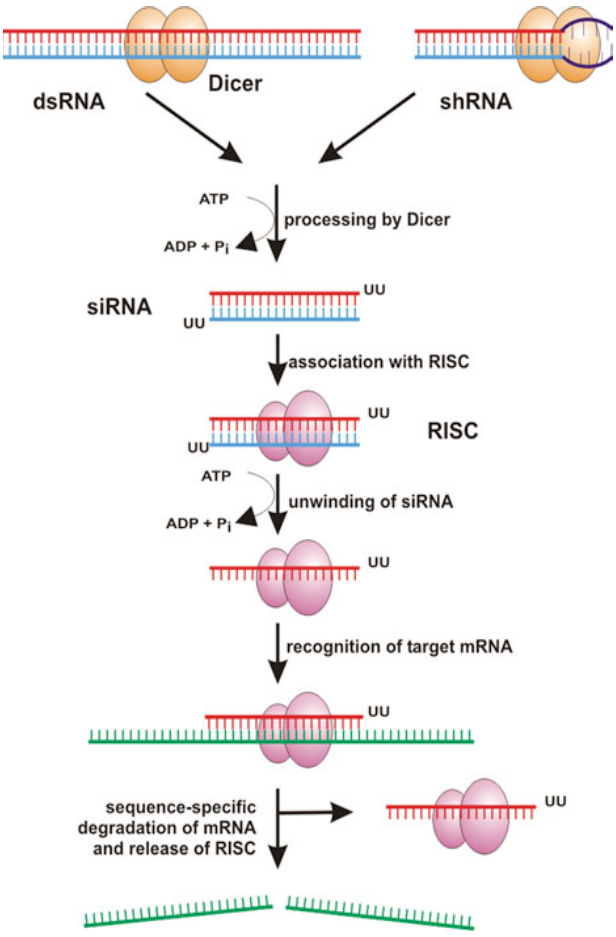

Figure 7: Mechanism of RNA inteference (Image modified from Rutz and Scheffold, 2004).

DsRNA: double-strand RNA; shRNA: short hairpin RNA; siRNA: small interfering RNA. See text for details.

Short-hairpin RNAs (shRNAs) are duplexes of RNA, encoded by plasmids or chemically synthesized and processed by the RNase III-like enzyme, DICER, into siRNA, e.g. RNA duplexes of 21–25 nucleotides in length with dinucleotide 3' overhangs. siRNAs are then incorporated into the RISC endoribonuclease (*R*NA-*i*nduced *s*ilencing *c*omplex). A helicase within RISC unwinds duplex siRNA allowing its antisense strand to bind a complementary mRNA. An RNase within RISC degrades the target mRNA by cleavage, which results in silenced gene expression and reduced protein production (Iorns *et al*., 2007) (Fig. 7).

In the last decade, RNAi has been developed as an efficient tool to obtain gene expression silencing; several iRNA libraries covering the entire coding transcriptome are available. iRNA libraries can be divided in two types: collections of vector-based shRNA expression vectors and libraries of siRNAs. siRNA reagents can be chemically syntesized or generated from cDNA by RNase III digestion (a method konwn as “esiRNA”) (Yang *et al*., 2002; Kittler *et al*., 2004). shRNA-based libraries are used to obtain a stable gene silencing because shRNA vectors integrate into genomic DNA; *viceversa*, libraries of siRNAs allow transient silencing of gene expression because siRNAs do not integrate in the host genome. siRNA libraries are currently used in two main formats, that are schematically described in figure 8.

In the arrayed screenings, cells are plated in multi-well plates already containing siRNAs and each gene is targeted separately to determine the phenotype of interest. Usually, the readout is cell viability (Fig. 8a). Alternatively, siRNAs complexed with trasfection reagents are plated onto slides as iRNA cell-microarrays and cells are trasfected directly on these arrays (Fig. 8b).

A third approach is the use of pooled shRNA libraries with which to transfect or infect cells, and then select for the phenotype of interest, such as resistance to a drug. The target gene in surviving cells is reveled by PCR amplification and by sequencing. A variant to this approach consists in the use of barcode screening: cells are trasfected with a pooled shRNA library and then divided in two populations, one treated with a selective agent (like a drug) and the other used as control. Each shRNA vector and shRNA control carry a specific barcode labelled with different fluorochromes; upon hybridization to a microarray containing a series of barcode-specific probes, the shRNA can be easily identified (Fig. 8c).

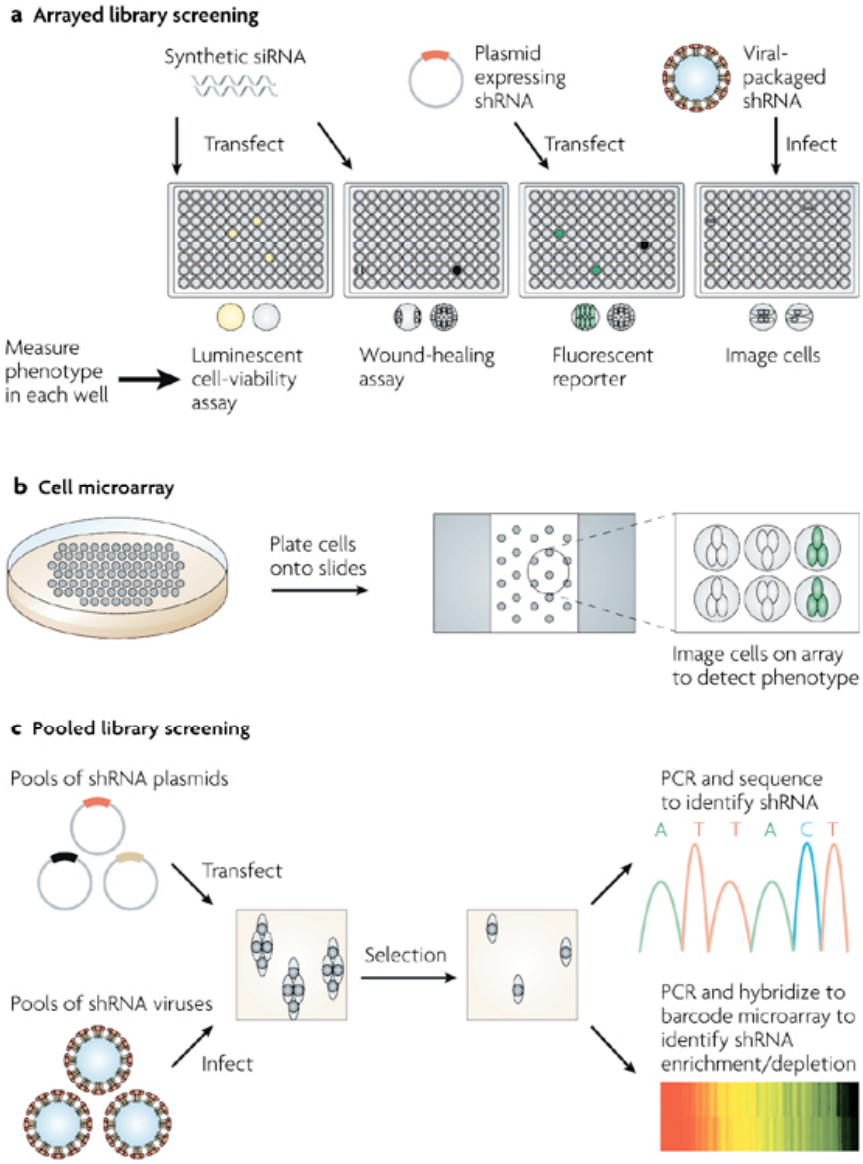

Figure 8: iRNA screening approachs (Image adapted from Iorns *et al*., 2007).

Transfection efficiency is generally quite high using siRNA libraries and significant silencing of gene expression is observed after three or four days, depending of experimental conditions; by contrast, the use shRNA-based libraries requires time and is more expensive. Appropriate positive and negative controls are necessary: negative controls should have no effect on gene silencing. For example, using cell lines that express a fluorescent protein such as *G*reen *F*luorescent *P*rotein (GFP), together with a siRNA that targets GFP would provide an ideal negative control for many screenings. The positive controls also provide an important threshold to define the magnitude of change that is thought to have a meaningful biological effect.

Thanks to the capability of the siRNA libraries to enable specific knock-down of entire gene families, several high-throughput screenings have been performed to identify new regulators of apoptosis and chemoresistance (MacKeigan *et al*., 2005), cell motility (Collins *et al*., 2006), proteasome function (Paddison *et al*., 2004) and mitotic progression (Moffat *et al*., 2006). RNAi screenings have been used to define the role of protein kinases in different cancer types (Grueneberg *et al*., 2008; Bommi-Reddy *et al*., 2008; Baldwin *et al.,* 2008).

2.3 Therapeutic targeting of oncogenic receptor tyrosine kinases

2.3.1 Tyrosine kinases family.

The sequencing effort of the Human Genome Project revealed that up the 20% of the almost 32,000 annotated genes encode proteins involved in signal transduction. Protein kinases of the human kinome are 518; approximately 90 are tyrosine kinases; among them, 32 are non-receptor and 58 receptor tyrosine kinases (RTKs), distributed in 20 subfamilies (Manning *et al*., 2002) (Fig. 9). Here we will discuss about tyrosine kinases and particularly receptor tyrosine kinases.

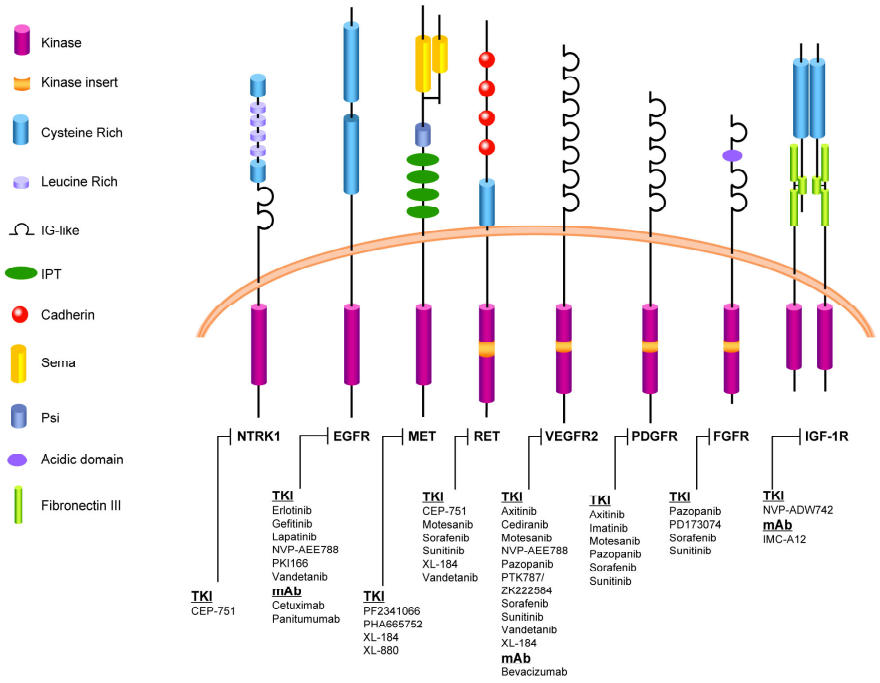

Figure 9: Receptor Tyrosine Kinase Families.

Some RTKs are shown with their proteins domains. Some of the small molecule as well as antibodies used to target these RTKs in cancer are also shown.

RTKs have a prominent role in many cellular functions, from cell growth to differentiation, migration, cytoskeleton rearrangement, apoptosis and cell cycle progression. There are three many principles regulating RTKs functioning (Lemmon *et al*., 2010). First, each cell type in the organisms expresses specific RTKs; second, the activation of RTKs is closely dependent on specific ligand availability and binding; third, signaling pathway involves intracellular target proteins activated through tyrosine phosphorylation by a given RTK.

The overall structure of RTKs is evolutionarily conserved from nematode *C. elegans* to humans. Each RTK is a transmembrane protein with three different regions:

- NH_2-terminal portion, containing a globular ligand-binding domain. This is the distinctive feature in the RTKs and is composed of a various recognizable sequence motifs; for example, *E*pidermal *G*rowth *F*actor *R*eceptor (EGFR) has two cysteine-rich regions, while the *E*rythropoietin *P*roducing-*H*epatocellular carcinoma cell receptor (EPH) subfamily has two fibronectin type III repeats, one cysteine-rich region and Ig-like motif; RET has four cadherin-like domains (Fig. 9).
- short α helix membrane-spanning, consisting of a stretch of hydrophobic residues that are followed by several basic residues that function as a stop-transfer signal;
- a juxtamembrane region followed by a cytoplasmic catalytic domain. The catalytic domain has a sequence of approximately 250 amino-acids highly conserved both in tyrosine and in serine/threonine kinases.

The catalytic domain is composed of two lobes: the N-terminal lobe contains a β-sheet followed by an α-helix and is responsible for binding Mg^{2+} /ATP at the motif GXGXXG. In the C-lobe, the sequence HRDLAARN forms the catalytic loop. Between them, there is a cleft allowing Mg^{2+} /ATP binding.

Unlike normal cells, where RTKs activation is tightly regulated, tumor cells often have a significant overexpression or mutation-dependent activation of RTKs. For this reason RTKs and/or their downstream effectors could be key targets of new specific anti-cancer drugs (Zhang *et al*., 2009). The first step in the signaling activation pathway of RTKs is the ligand binding to the extracellular receptor domain (Lemmon *et al*., 2010). Downstream events can be summarized as follows:

1. Receptor dimerization: even though early studies of RTKs have suggested as simplest mechanism of receptor dimerization a "ligand-mediated" model, subsequent studies provided important insights into additional mechanisms of receptor dimerization. Apart the case of NTRK1 whose dimerization is realized in ligand-mediated manner without any interaction between extracellular domains of each receptor molecule, three other modes of dimerization are possible. Dimerization is "receptor-mediated" when the ligand makes no direct contribution to the event. This happens in EGFR family. A second possibility is ligand-mediated dimerization with few receptor contacts, as for KIT receptor. Finally, a third possibility exemplified by FGFR (*F*ibroblast *G*rowth *F*actor *R*eceptor), happens when receptor molecules contact each other through the Ig-like domain D2 and the accessory molecules of heparin and *h*eparin-*s*ulfate *p*roteo*g*lycans (HSPG) also interact with this domain.

2. Activation of Intracellular kinase domain: the activation of intracellular catalytic domain is the crucial point of signaling transduction pathway. RTKs activation is regulated by an autoinhibition mechanism (Hubbard, 2004). Briefly, the activation loop interacts directly with the active site of catalytic domain and blocks access to protein substrates and ATP; thus, the tyrosine kinase domain is autoinhibited by a *cis*-autoinhibitory interaction. After growth factor binding, a specific tyrosine residue ("Y") in the catalytic domain is phosphorylated by its partner ("*trans*-phosphorylation"), eliminating *cis*-autoinhibition and allowing tyrosine kinase domain to adopt the active state.

3. Activation of intracellular signaling pathway via target proteins recruitment. Signaling by an activated RTK is mediated by phosphorylation or binding of citoplasmic proteins that in turns activate downstream targets. There are three mechanisms underlying activation of intracellular cascade mediated by RTKs. The first involves enzymes directly phosphorylated by an activated RTK like PLCγ, RasGAP or Src kinases. The second involves proteins lacking an intrinsic catalytic activity called "adaptors" that serve as intermediates between activated RTK and downstream molecules. These proteins contain *S*rc *h*omology *2* (SH2) or *P*rotein *T*yrosine-*B*inding (PTB) domains that specifically bind to phosphotyrosine (Pawson, 2004). They can be recruited directly by receptor phosphotyrosines or indirectly by binding to docking proteins phosphorylated by RTKs. Finally, some targets of RTKs are structural proteins whose phosphorylation is responsible for the rapid membrane and cytoskeletal rearrangement occurring after RTK activation.

2.3.2 Receptor tyrosine kinases and cancer.

There are four main principles for oncogenic transformation of RTKs (Lemmon *et al*., 2010).

1. Retroviral transduction: in 1983, it was observed that the v-*sis* oncogene from simian sarcoma virus originated by viral trasduction of the PDGF gene (Doolittle *et al*., 1983; Waterfield *et al*., 1983) and that its protein product ($p28^{sis}$) promoted cell transformation by activating PDGFR in an autocrine loop.

2. Genomic rearrangement, as in the case of RET/PTC oncoproteins (Fig. 3).

3. Oncogenic mutations arising from point mutations, deletions or substitutions in cytoplasmic or transmembrane tyrosine kinases. A good example is RET in MTC (Fig. 6).

4. Gene amplification: an important example is the amplification of genes coding for Neu/ErbB2 and EGFR in breast and lung cancer, respectively.

2.3.3 Receptor tyrosine kinases as target for cancer therapy

The past two decades have wittnessed significant progress in understanding cancer pathogenesis. Thus, actual efforts are focusing on development of anti-cancer drugs that target proteins involved in carcinogenesis by which it will be possible to kill tumor cells while sparing normal cells.

Several drugs have been approved by the US Food and Drug Administration (FDA) and European Authorities to treat cancers and other diseases caused by activated RTKs (Fig. 9) (Zhang *et al*., 2009). These drugs fall in two groups: small-molecules inhibitors targeting the ATP-binding site of the intracellular receptor kinase domain (Shawver *et al*., 2002) and monoclonal antibodies that either interfere with RTK activation or target RTK-expressing cells for destruction by the immune system (Reichert and Valge-Archer, 2007). In most cases, tyrosine kinase inhibitors target the ATP-binding site of protein kinases, thus inhibiting multiple kinases in addition to their initially intended targeted (Zhang et al., 2009). Gleevec (Imatinib mesylate) is the prototype of these compounds, acting as inhibitor of BCR-ABL fusion protein in CML (*C*hronic *M*yeloid *L*eukemia), and of PDGF receptors α and β and c-KIT in GIST (*G*astro-*I*ntestinal *S*tromal *T*umors). Gefitinib (Iressa) and Erlotinib (Tarceva) are EGFR inhibitors used to treat non-small-cell lung cancer in case of EGFR mutation, overexpression or both (Pao *et al*., 2004). Sunitinib (Sutent) and Sorafenib block tyrosine kinase activity of several RTKs, such as KIT, VEGFR2, PDGFR and RET and have been successfully applied to treat GIST and renal cell carcinoma (Chow and Eckhardt, 2007).

Several compounds able to target the RET kinase including Sunitinib, Sorafenib, Vandetanib and XL-184 are in clinical studies in patients with thyroid cancer (Santoro *et al*., 2006; Schlumberger *et al*., 2009; Sherman, 2009) (Fig. 9).

2.3.4 Acquired resistance to tyrosine kinase inhibitors.

There are two molecular mechanisms causing cancer resistance to TK drug treatment. First, amplification or point mutation in the oncogenic protein kinase. Resistance to TK-targeted therapy was first identified in patients with advanced form of CML resistant to Imatinib therapy and associated to several point mutations in BCR-ABL (Zhang *et al*., 2009). Resistance to Gefitinib in patients with lung adenocarcinoma is mediated by T790M mutation in EGFR (Bean *et al*., 2008; Sharma *et al*., 2005). In this frame, "second-generation" inhibitors were developed to overcome mutations that cause resistance to the first inhibitor. Second-generation ABL kinase-inhibitors are successfully used in the treatment of CML cases bearing BCR-ABL mutants resistant to Imatinib (Shah *et al*., 2004). Similarly, in GIST with acquired KIT mutations conferring resistance to Imatinib, Sutent (Sunitinib) has shown significant activity (Demetri *et al*., 2009).

Alternatively, acquired resistance to an inhibitor may result from the activation of alternative kinase(s) that rescue cancer cell proliferation; as an example in breast cancer, resistance to ErbB2-targeted therapy can be mediated by overexpression of MET receptor (Shattuck *et al*., 2008). In order to overtake this mechanism of resistance, e.g. the kinase switch, it is necessary to identify the compensating kinase(s) and to develop tools to inibit it(them); siRNA screen of the kinome in cancer cells can be exploited to achieve this goal.

2.4 EPH receptors and ephrins.

The largest group of RTK is made up of EPH family. In 1987 the first EPH receptor was identified during a screening looking for new tyrosine kinases involved in human cancer. The receptor was named EPH, after the *E*rythropoietin *P*roducing *H*epatocellular carcinoma cell line from which its cDNA was cloned (Hirai *et al*., 1987). Initially, all EPH were considered as orphan receptors because the absence of ligands already known. The first ligand was identified in HUVEC cells (*H*uman *U*mbilical *V*ein *E*ndothelial *Cells)* (Holzman *et al*., 1990) as a novel TNFα (*T*umor *N*ecrosis *F*actor-α)-inducible gene product and named ephrinA1; few years later it was established ephrinA1 as a ligand of EphA2 (Bartley *et al*., 1994). To date, 16 EPH receptors are known in vertebrates, 14 of them in mammals (Gale *et al*., 1997).

Based on sequence homology of the extracellular domain and ability to interact with a particular ephrin ligand, EPHs are classified in A and B types. The ligands are named ephrins (*Eph-r*eceptor *in*teracting proteins, shortly EFN) and similarly are divided in two subclasses based on their structural features. Nine type-A and five type-B receptors interact with ephrins-A and ephrins-B, respectively. Binding between receptors and ligands of the same class is highly promiscuous (Pasquale *et al.,* 2004); exceptions are EphA4 that binds ephrins-B, EphB2 that binds ephrin-A5, and EphB4 that interact with ephrin-B2 only (Pasquale *et al.,* 2004).

Type-A (ephrinA1 to A5) and type-B (ephrinB1 to B3) ephrins share a conserved core sequence of approximately 125 amino acids, including four invariant cysteine residues, probably corresponding to receptor binding domain. This core is followed by a membrane anchor-domain, a GPI-anchor for A-ligands and a transmembrane domain for B-ligands (Fig. 10).

Similar to others RTK, EPHs have an extracellular portion with a ligand binding domain, a cysteine rich-domain (that contains an EGF-like motif) and two fibronectin-type III repeats. Cytoplasmic region has a typical juxtamembrane portion with two invariant tyrosine residues (Y596 and Y602 of EphA4; Y604 and Y610 of EphB2) that are embedded in highly conserved sequence motif of almost ten amino-acids. These residues undergo autophosphorylation after receptor activation and are crucial for activation of downstream interacting proteins. The tyrosine kinase domain is followed by a COOH-terminal that includes a SAM (*S*terile α *M*otif) and a PDZ motif (*P*SD-95/-*D*isc large-*Z*ona occludens tight junction protein) (Fig. 10).

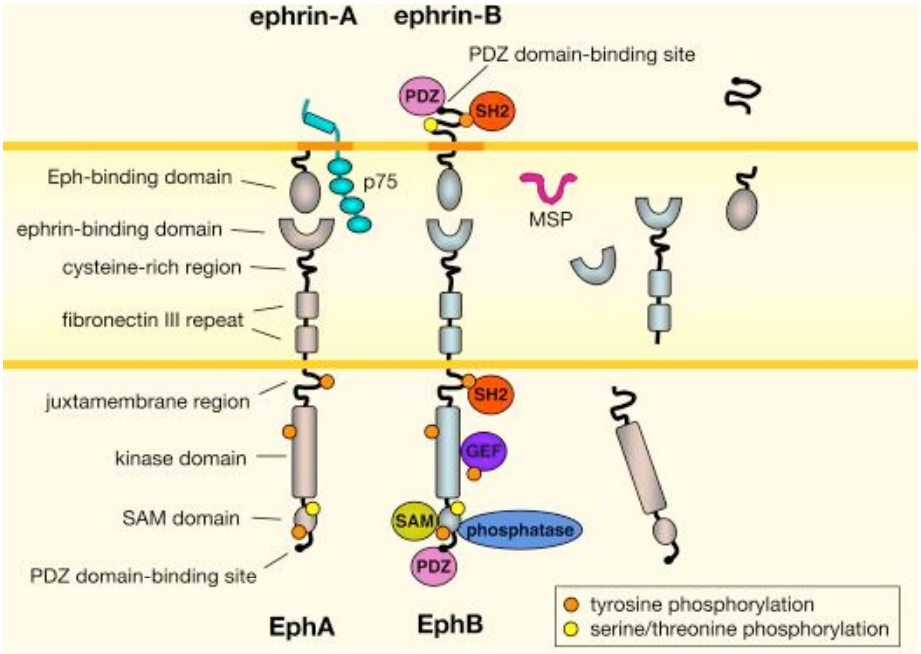

Figure 10: Structure of EPH receptors and their ligands with signaling interactions.

Tyrosine (orange rings) or serine/threonine (yellow rings) phosphorylation and downstream interactors with SH2, SAM and PDZ domains are shown. The MSP (*M*ajor *S*perm *P*rotein) is another EPH ligand that can compete with ephrins for binding. Ephrin-B also mediate a "reverse" signaling through interactors containing SH2, SAM and PDZ domains. Phosphate groups are removed from tyrosines by phosphatases that prevent continuous receptor activation (Pasquale, 2005).

EphA10 and EphB6 lack residues for catalytic activity, indicating that these two receptors might not function as kinases and phosphorylate cytoplasmic target proteins (Murai *et al*., 2003).

Given that both EPH receptors and ephrins are membrane-bound, their interaction occurs only at sites of cell-cell contact. In the absence of interaction, they exist in loosely associated microdomains, which become more compact and well-ordered when EPH-ephrin complexes assemble to generate clearly defined signaling centers (Pasquale, 2005). Otherwise the majority of cell surface receptor-mediated signal trasduction systems, EPHs and ephrins send information bidirectionally, that is: activated EPH transduces a "forward" signal into the receptor-bearing cell, whereas, at the same time, ephrin activates a "reverse" signal into the ephrin-expressing cell (Gale *et al*., 1996).

EPHs are involved in a wide range of biological functions, both in developing and mature structures and are strongly expressed in nervous system where they provide paths for axon guidance (Canty *et al*., 2006; Coulthard *et al*., 2002; Dottori *et al*., 1998), regional migration of neural crest cells (Krull *et al*., 1997), glioma cell proliferation (Fukai *et al*., 2008) and development of neuromuscolar junctions (Lai *et al*., 2001). Moreover, during synaptogenesis EPHs help establish (Dalva *et al.,* 2000) and modify the postsynaptic specialization (Ethell *et al*., 2001) by trasmitting signals to the actin cytoskeleton through the Rho-family of small GTPases (Irie and Yamaguchi, 2002). EPHs and ephrins also play an important role in the organization and function of non-neural tissue; as an example, both EphBs and EphAs and their ligands are widely expressed in the arterial and venous endothelium and between endothelial cells and the surrounding mesenchyme (Kuijper *et al*., 2007).

2.4.1 Mechanisms of EPH-ephrin signaling.

The first step of EPH signaling is the monovalent interaction between a receptor and a ligand on juxtaposed cells, inducing conformational rearrangement in both of them. In addition to the high-affinity binding site, the receptor and the ligand have also a low-affinity binding interface which can mediate the dimerization of two EPHs-ephrins dimers, leading to a tetrameric structure formation. EPH-ephrins complexes progressively aggregate into larger clusters the size of which depends of their density on cell surface. After interaction, specific tyrosine residues in each EPH kinase domain undergo *trans*-phosphorylation (Binns *et al*., 2000; Huse and Kuriyan, 2002), promoting kinase activity by disrupting intramolecular inhibitory interactions that occur between the juxtamembrane segment and kinase domain, converting it into its active state.

2.4.2 Forward signaling.

EPH class-A receptors signal through Rho-family small GTPases (Rho, Rac and Cdc42), regulating actin dynamics (Hiramoto-Yamaki *et al*., 2010) (Fig. 11, ***left***), whereas class-B receptors interact with Cdc42 through Intersectin (Irie and Yamaguchi, 2002) and Kalirin (Penzes *et al*., 2003) (Fig.11, ***right***). Ras family activity is negatively regulated by EPHs (Pratt and Kinch, 2002). EPHs can also influence other signaling pathways, such as FAK (*F*ocal *A*dhesion *K*inase) in the case of EphAs (Miao *et al*., 2000), and LMW-PTP (*L*ow-*M*olecular *W*eight *p*hospho*t*yrosine *p*hosphatase) in the case of EphBs (Fig. 11).

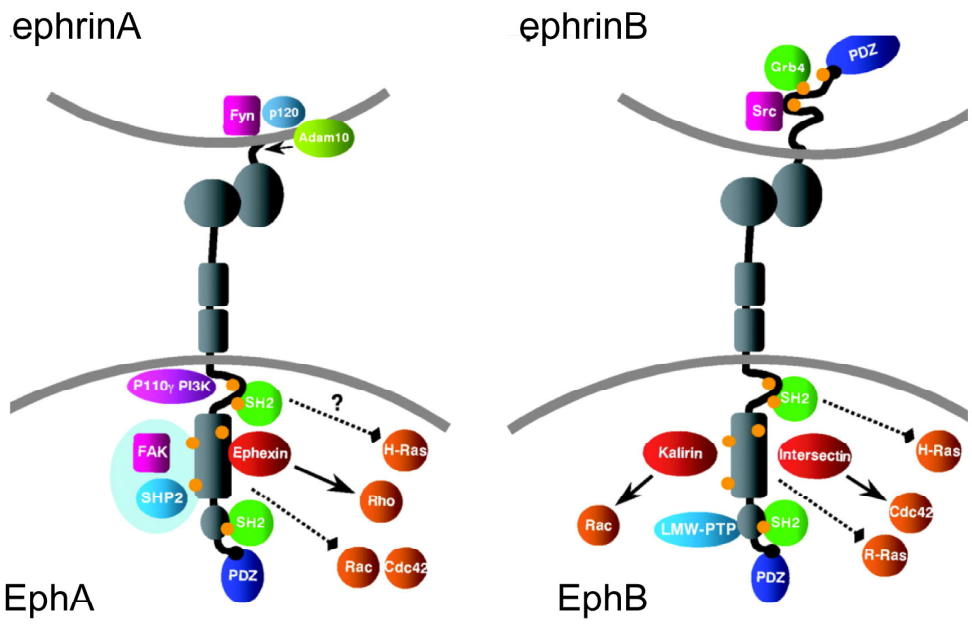

Figure 11: Forward and reverse signals downstream of the Eph-ephrin complexes (Murai *et al.*, 2003).

2.4.3 Reverse signaling.

The first study suggesting the existence of reverse signaling dates middle 1990s (Brambilla *et al.*, 1995). Ephrin-B transmit a reverse signal trough Grb4, Src family kinases (Holland *et al.*, 1997; Palmer *et al.*, 2002) and PDZ-RGS3 (Lu *et al.*, 2001). Since ephrins-A are membrane-bound through GPI-anchor, they activate reverse signaling recruiting Src family members like Fyn and a 120 KDa lipid raft protein (Davy *et al.*, 1999; Huai and Drescher, 2001) to the lipid rafts. EphrinA-mediated reverse signaling is regulated by Adam10 metalloprotease (Janes *et al.*, 2005), which cleaves the ligand from cell surface. This cleavage has a dual function: first, it allows the EPH-bearing cell to change its response to ligand and secondly it allows to terminate both reverse and forward signaling.

2.4.4 EPHs as potential therapeutic target.

In several human cancers, EPHs are overexpressed or functionally active. EphA2 overexpression is associated to poor prognosis in many cancers (Ireton *et al*., 2005; Landen *et al*., 2005; Wyosky and Debinski, 2008). However, it should be also mentioned that in other cases EPH seem to undergo loss-of-function. EphA2, EphA8 and EphB2 are clustered in 1p36, that frequently undergoes to LOH in neural crest tumors (Sulman *et al*., 1997). EphA1 and EphB2 are downregulated in advanced colorectal cancer (Herath *et al*., 2010), EphA3 is downregulated in leukemias and hematopoietic tumor cells (Dottori *et al*., 1999) and EphA7 in prostatic, gastric and colorectal cancer (Guan *et al*., 2009). However, since in most of the cases a gain of EPH function has been found in cancer, several therapeutics possibilities have been developed to inhibit EPH. EPH competitors, as XL67 (orally available EGFR and VEGFR inhibitor targeting also EphB4), are being evaluated in clinical trials for lung cancer. Antibodies, decoy receptors, oligonucleotides and siRNAs are used to induce EphA2 and EphA4 mRNA or protein downregulation (Carles-Kinch *et al*., 2002, Duxbury *et al*., 2004; Landen *et al*., 2005).

Recently, two 2,5-dimethylpyrrolyl benzoic acid derivatives have been identified as selective inhibitors of EphA2 and EphA4 (Noberini et al., 2008). These two compounds block receptor-efn binding and may be promising leads to treat cancers caused by dysregulation of EphA2 and EphA4 function.

3. AIM OF THE STUDY

The aim of this study was to identify new kinases regulating thyroid cancer cell proliferation. The specific aims were as follows:

1) To identify through a RNA interference-based screening new kinases whose knock-down significantly affect thyroid cancer cell viability.

2) To validate these hits by an independent set of siRNA.

3) To perform functional assays in order to clarify the role of the identified kinases in controlling proliferation of thyroid cancer cells.

4) To study the role exerted by members of EPH family in thyroid cancer cells.

4. MATERIALS AND METHODS

4.1 siRNA-based screening of the human kinome.

The primary screening was performed with the human kinome siRNA set v 2.0 (Qiagen) in a 384-well microtiter plate format. Each well contained a specific siRNA targeting one of 646 distinct genes (518 protein kinases and 128 kinase-related genes). Two siRNAs for each target were used. For high-throughput transfection, 5 µl of 2 micromolar siRNA (10 pmol) were transferred from the stock plate using a robotic liquid handler (ThermoLabsystems). Transfection solution was prepared with 0.2 µl of HyPerfect® Transfection reagent (Qiagen) diluted in 10 µl of Optimem medium (Invitrogen). Following 10 minutes of incubation, the transfection mix was delivered to each well with a MultiDrop dispenser (ThermoLabsystems) and incubated at room temperature for 1 hour. All wells were then seeded with 1,000 TPC1 cells/well in a volume of 100 µl taken from a single-cell suspension in DMEM (*D*ulbecco's *m*odified *E*agle's *m*edium) medium (Invitrogen) and 2.5% FBS (*f*etal *b*ovine *s*erum) without antibiotics.

4.2 Cell Viability measurement.

Seventy-two hours after transfection, Cell Titer Blue reagent (CellTiter® Blue Assay, Promega) diluted 1:1 in cell culture medium was added to each well. Plates were incubated at 37 °C for 6 hours with 5% CO_2 and then transfered to room temperature for overnight incubation, at dark.

Cell viability was measured with an EnVision Multilabel plate reader (Perkin Elmer). Transfection efficency was previously tested by using the GAPDH Alert kit (Ambion) according to the manifacturer's protocol.

4.3 Cell lines.

Human primary cultures of normal thyroid cells (P5) were obtained from F. Curcio and cultured as previously described (Curcio *et al*., 1994). Human papillary (TPC1, BCPAP, NIM) and anaplastic (BHT101, OCUT1, CAL62, 8505C, SW1736) thyroid cancer cell lines have been described previously (Salerno *et al*., 2010). All the cells were SNP genotyped to ensure correct identity. NTHY (Nthy-ori 3-1) are normal human thyrocytes immortalized by the Large T of SV40 and were obtained from the European Tissue Culture collection. NTHY and tumor cell lines were grown in the DMEM medium supplemented with either 2.5% or 10% FBS, L-glutamine and penicillin/streptomycin (all reagents were from Sigma, Munich, Germany).

4.4 Tissue Samples.

A small set of PTC, ATC and normal thyroid tissue samples snap-frozen in liquid nitrogen and maintained at -80°C has been made available by F. Basolo (University of Pisa, Italy). For all of them, formalin-fixed paraffin-embedded tissue slides were reviewed by 2 pathologists (F. Basolo; C. Ugolini) to ensure diagnosis (Hedinger *et al*., 1989). RNA was extracted with the RNeasy mini kit (Qiagen) according to the manifacturer's instructions.

4.5 Quantitative RT-PCR.

For evaluation of mRNA expression levels, cell lines were grown to 70% confluency in DMEM with 10% FBS, and then total RNA was extracted with RNeasy mini kit (Qiagen) and subjected to on-column DNase digestion with the RNase-free DNase set (Qiagen) according to the manufacturer's instructions. Random-primed first strand cDNA was synthesized in a 50 µl reaction volume starting from 2 µg RNA by using the GeneAmp RNA PCR Core Kit (Applied Biosystems). PCR amplification was performed using the GeneAmp RNA PCR Core Kit system starting from 2.5 µl of RT product in a reaction volume of 25 µl according to the manufacturer's instructions. Primers were designed by using a software available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi and synthesized by the CEINGE (Naples, Italy). Quantitative (real-time) reverse transcription polymerase chain reactions (qRT-PCR) were performed by using the SYBR Green PCR Master mix (Applied Biosystems) in the iCycler apparatus (Bio-Rad). Amplification reactions (25µl final reaction volume) contained 200 nM of each primer, 3 mM $MgCl_2$, 300 µM dNTPs, 1x SYBR Green PCR buffer, 0.1U/µl AmpliTaq Gold DNA Polymerase, 0.01U/µl Amp Erase, RNase-free water, and 2 µl cDNA samples. To verify the absence of non-specific products, 80 cycles of melting (55°C for 10 sec) were performed. In all cases, the melting curve confirmed that a single product was generated. Amplification was monitored by measuring the increase in fluorescence caused by the SYBR-Green binding to double-stranded DNA. Fluorescent threshold values were measured in triplicate and fold changes were calculated by the ΔΔCt formula: $2^{-(\text{sample 1 } \Delta Ct \text{ - sample 2 } \Delta Ct)}$, where ΔCt is the difference between the cycle threshold (Ct) of the mRNA of interest and the Ct of the *β*-actin mRNA used as housekeeping gene.

4.6 Protein studies.

Harvested cells were lysed in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl2, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and clarified by centrifugation at 10,000Xg at 4°C. Protein concentration was estimated with a modified Bradford assay (Bio-Rad Laboratories).

4.7 Human Phospho-RTK arrays.

The relative levels of tyrosine phosphorylation of receptor tyrosine kinases (RTKs) were evaluated with a Human Phospho-RTK array kit (R&D Systems, Inc.). Briefly, harvested cells were lysed in NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% Glycerol, 2 mM EDTA, 1 mM Sodium Orthovanadate, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin) and centrifuged at 14,000Xg for 5 minutes at 4°C. For each array, 500 μg of lysate were used. Each array contained 42 different anti-RTK antibodies and six controls printed in duplicate. Non specific binding was blocked for 1 hour at room temperature on a rocking platform shaker. The lysates were diluted in Array Buffer 1 (provided with the kit) and added to each array for overnight incubation at 4°C. Freshly diluted detection antibody was added to the arrays for 2 hours of room temperature incubation. Phosphorylation levels were detected by chemiluminescence (ECL; Amersham Biosciences).

4.8 BrdU Assay.

BrdU (5-bromo-2-deoxyuridine) incorporation assay was performed by using the Cell Proliferation ELISA-BrdU chemiluminescent Kit (Roche Applied Science). Transfected cells were labeled with BrdU, then fixed and incubated with anti-BrdU-Peroxidase solution. Finally, each sample was incubated with a buffered solution containing luminol and BrdU incorporation was quantified by measuring light emission with a microplate luminometer with photomultiplier technology.

4.9 Apoptosis Assay.

The Apo-ONE® Homogeneous Caspase-3 Assay (Promega) was performed in thyroid cancer cell lines upon siRNA transfection. Blank, positive and negative controls were performed as well. Apo-ONE® Homogeneous Caspase-3 Reagent was added 1:1 to cell culture volume. The fluorescence values were measured with an EnVision Multilabel plate reader (Perkin Elmer), proportionally to the amount of caspase-3/7 cleavage activity in each sample.

4.10 Chemoinvasion Assay.

In vitro invasiveness through Matrigel was assayed using transwell cell culture chambers. Briefly, confluent cell monolayers were harvested with trypsin/EDTA and centrifuged at 800X*g* for 10 minutes.

The cell suspension ($1X10^5$ cells/well) was added to the upper chamber of transwells on pre-hydrated polycarbonate membrane filter of 8 μM pore size (Costar) coated with 35μg Matrigel (Collaborative Research Inc.). The lower chamber was filled with complete medium. Cell dishes were incubated at 37 °C in 5% CO_2 and 95% air for 24 hours. Non-migrating cells on the upper side of the filter were wiped off and migrating cells on the reverse side of the filter were stained with 0.1% crystal violet in 20% methanol for 15 minutes, counted and photographed.

4.11 Wound Healing Assay.

A wound was induced on the confluent monolayer cells by scraping a gap using a micropipette tip. Photograps were taken at 100X magnification using phase-contrast microscopy immediately after wound incision and 12 hours later.

4.12 Statistical analysis.

The raw data of the siRNA screen were normalized and analyzed with the R/Bioconductor software (Gentleman *et al*. 2004).

5. RESULTS

5.1 High-throughput identification of human kinases required for TPC1 cells viability.

We designed a functional RNAi-based screening using the human papillary carcinoma cell line, TPC1, harboring the RET/PTC1 rearrangement (Schweppe *et al*., 2008). A commercial siRNA library containing 646 synthetic siRNAs targeting all human protein kinases (518) and some kinases-related and associated proteins was used. To reduce the off-target activity of siRNAs, the library has been designed to contain multiple (at least 2) independent duplexes targeting the same transcript. To monitor transfection efficiency, each plate contained also siRNAs targeting genes that are common positive regulators of proliferation of every mammalian cell type (e.g. *p*olo *l*ike *k*inase 1, PLK1). Negative control siRNAs, targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH), green fluorescent proteine (GFP) and scrambled non targeting sequences, were also used (Fig. 12).

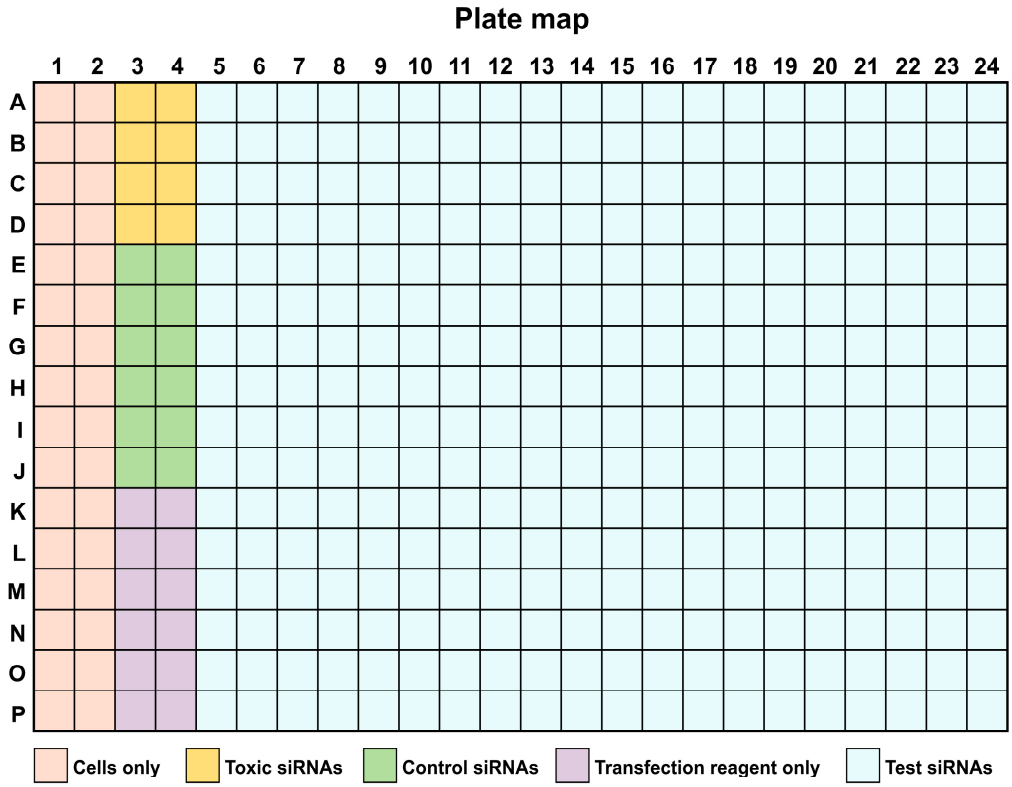

Figure 12: Kinome screen plate map.

Positions of controls and test wells used for primary screen assay are shown.

5.2 Identification of primary hits.

Initially, transfection conditions for TPC1 cells were set up by using the GAPDH Alert kit (Ambion). Subsequently, the screening was performed in duplicate. Seventy-two hours after transfection, a CellTiter blu assay was performed to stain viable TPC1 cells in order to identify genes (coding for kinases and related proteins, hereafter referred to as “hits”) whose silencing affected TPC1 cell viability.

Primary hits were defined as genes that, when silenced, reduced cell viability by at least 30%. Hits that did not confirm in both duplicates were excluded from further analysis. Down-regulation of approximately 6% of the kinome library (41 hits) resulted in reduced TPC1 cell viability. A secondary validation screen was then performed with an independent set of two additional different siRNAs targeting a distinct mRNA region of the highest ranked 41 hits. Twenty-one hits were confirmed (e.g. caused at least 30% reduction of cell viability) and were further studied. Figure 13 shows the flow-chart of the screening.

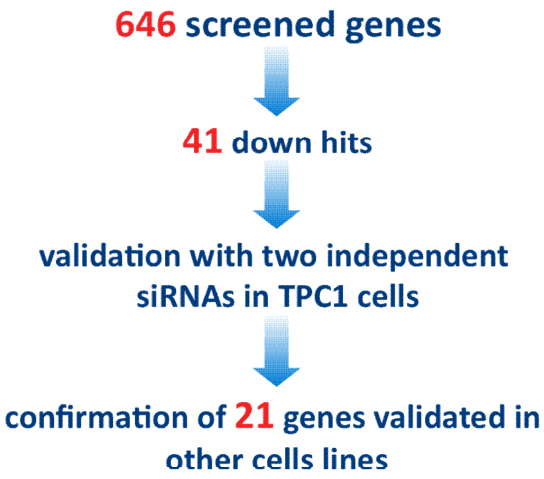

Figure 13: Flow chart of the kinome siRNA screening in TPC1 cells.

Table 2 summarizes the list of the 21 hits. They included expected candidates, like cell cycle kinases (STK33) and signaling kinases (FYN and AKT2), together with some new kinases that have not previously associated to thyroid carcinogenesis (Table 2).

Symbol	Full name	Subgrup	GenbankID	siRNA1	siRNA2
AKAP9	A kinase (PRKA) anchor protein 9	KAP	NM_005751		
AKT2	v-akt murine thymoma viral oncogene homolog 2	AGC	NM_001626		
EPHA2	EPH receptor A2	TK	NM_004431		
EPHA4	EPH receptor A4	TK	NM_004438		
EPHA5	EPH receptor A5	TK	NM_004439		
EPHA7	EPH receptor A7	TK	NM_004440		
EPHB2	EPH receptor B2	TK	NM_017449		
EPHB6	EPH receptor B6	TK	NM_004445		
FYN	FYN oncogene related to SRC, FGR, YES	TK	NM_002037		
GRK4	G protein-coupled receptor kinase 4	other	NM_182982		
HCK	hemopoietic cell kinase	TK	NM_002110		
LIMK1	LIM domain kinase 1	TKL	NM_002314		
MAP3K6	mitogen-activated protein kinase kinase kinase 6	STE	NM_004672		
MAP3K7IP1	mitogen-activated protein kinase kinase kinase 7 interacting protein 1	KAP	NM_006116		
MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1	STE	NM_007181		
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase 2	STE	NM_004759		
PKN1	protein kinase N1	AGC	NM_002741		
PRKD2	protein kinase D2	CAMK	NM_016457		
RPS6KA6	ribosomal protein S6 kinase, 90kDa, polypeptide 6	AGC	NM_014496		
SMG1	PI-3-kinase-related kinase SMG-1	atypical	NM_001896		
STK33	serine/threonine kinase 33	CAMK	NM_030906		

Table 2: Genes whose silencing reduces TPC1 cell viability.

A color scale is reported to show the intensity of the effects.

The down-regulation efficiency of the target transcript, by the two additional siRNA used for the validation screen, was determined by quantitative RT-PCR. A scrambled control and non-transfected cells were used as reference. As shown in Figure 14, in TPC1 cells all 21 hits were significantly depleted by their cognate siRNAs, with the exception of only few poorly efficient siRNAs.

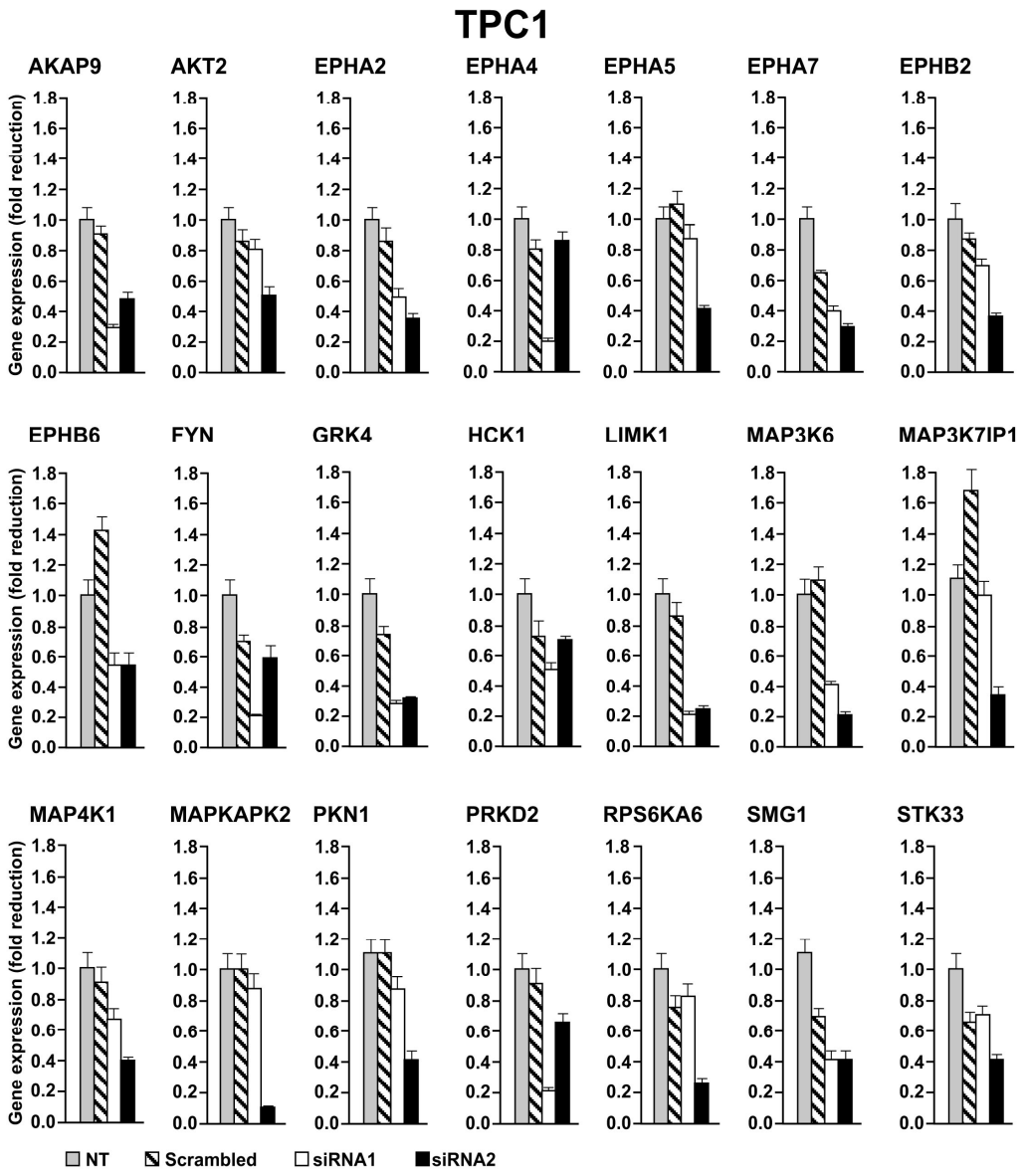

Figure 14: siRNA-mediated silencing of the 21 hits.

TPC1 were transiently transfected with the two different siRNA against the 21 identified hits. After 72 hours, RNA was extracted and mRNA expression levels were measured by Q-RT-PCR. Results are reported as fold reduction in comparison to the scrambled control.

5.3 Confirmation screen.

We asked whether the 21 identified hits were involved in the viability of thyroid cancer cell lines other than TPC1. To assess this point, we performed a confirmation screen by transfecting the two siRNA of the library in three additional thyroid cancer cell lines (BCPAP, 8505C, Cal62) (Schweppe *et al*., 2008). Table 3 summarizes the genetic features of the thyroid cell lines used in this and in next experiments.

Name	Type	Oncogene	P53	Other mutations
NIM	PTC	BRAF V600E (heteroz)	—	—
TPC1	PTC	RET/PTC1	—	—
BCPAP	PTC	BRAF V600E (heteroz)	D259Y	—
CAL62	ATC	KRAS G12R	A161D	Del157p14ARF
BHT101	ATC	BRAF V600E (heteroz)	I251T	
8505C	ATC	BRAF V600E (homoz)	R248G	Del1-150 p14ARF
SW 1736	ATC	BRAF V600E (heteroz)	Low expression	—
OCUT-1	ATC	BRAF V600E (heteroz)	—	—

Table 3: Genetic characteristics of thyroid cancer cell lines used in the study.

BCPAP is a PTC cell line harboring the BRAF V600E mutation; 8505C and Cal62 are ATC cell lines harboring the BRAF V600E and the KRAS G12R mutations, respectively (Schweppe *et al*., 2008). Of note, BCPAP, Cal62 and 8505C harbor also P53 mutations (Schweppe *et al*., 2008). Importantly, in our laboratory all these cell lines were SNP genotyped to ensure their identity (Salerno *et al*., 2010).

We also asked whether the 21 hits were necessary for the viability of normal thyroid cells. To this aim, we used two normal thyroid cell lines (NTHY and P5). P5 is a primary culture of normal human thyrocytes (Curcio *et al*., 1994), while NTHY (Nthy-ori 3-1) are normal human thyrocytes immortalized by the Large T of SV40.

A siRNA transfection experiment was performed on these additional 5 cell lines and results are listed in Table 4. Virtually, all the siRNAs reduced the viability of at least one of the thyroid cancer cell lines, while they had no effect on normal cells. The only notable exception was STK33 kinase that reduced the viability of normal cells, as well.

	TPC1	BCPAP	8505C	CAL62	NTHY	P5
AKAP9	+	+	+	+	-	-
AKT2	++	+	++	+	-	-
EPHA2	++	+	+	+	-	-
EPHA4	+	+	+	+	-	-
EPHA5	+	+	-	-	-	-
EPHA7	+	+	+	+	-	-
EPHB2	+	+	+	+	-	-
EPHB6	++	+	++	+	-	-
FYN	+	+	+	+	-	-
GRK4	++	+	++	+	-	-
HCK	++	++	-	-	-	-
LIMK1	+	-	-	-	-	-
MAP3K6	+	-	-	-	-	-
MAP3K7IP1	+	++	-	-	-	-
MAP4K1	++	+	+	+	-	-
MAPAPK2	+	+	+	+	-	-
PKN1	++	+	-	-	-	-
PRKD2	+	+	-	-	-	-
RPS6KA6	+	+	+	+	-	-
SMG1	++	+	++	+	-	-
STK33	++	++	+	+	+	+

Table 4: siRNA that reduce viability of the various thyroid cell lines.

++: ≥50% cell viability inhibition; +: 30-50 % cell viability inhibition; -: ≤ 30% inhibithion.

In particular, we could identify some hits (LIMK1 and MAP3K6) that were important for cell viability of TPC1 cells only, suggesting their effect might be probably linked to the presence of RET/PTC1. Moreover, other hits, namely EPHA5, HCK, MAP3K7IP1, PKN1 and PRKD2 were required for the viability only of PTC cells (TPC1 and BCPAP), but not ATC cells (8505 and CAL62).

5.4 Biological effects of the 21 hits knock-down in thyroid cells.

To better characterize the biological effect of the knock-down of the 21 hits in thyroid cells, we performed cell count and DNA synthesis measurement (BrdU assay) for cell proliferation as well as caspase-3 cleavage assay for cell apoptosis detection upon siRNA transfection. We used TPC1, BCPAP and NTHY cells. Transfection of siRNAs against all of the 21 hits decreased number of TPC1 cells on average by 40-50% (Fig. 15). Moreover, all of the 21 siRNAs decreased BrdU incorporation on average by 30-40% in the TPC1 and by 20-30% in BCPAP but not in normal cell lines. Only STK33 resulted essential for both cancer and normal cells (Figs. 16-17). No significant cytotoxic effect (measured as caspase 3 cleavage) was observed, with the notable exception of STK33 siRNA that had cytostatic and cytotoxic effects in all the cells (cancer and normal) (data not shown).

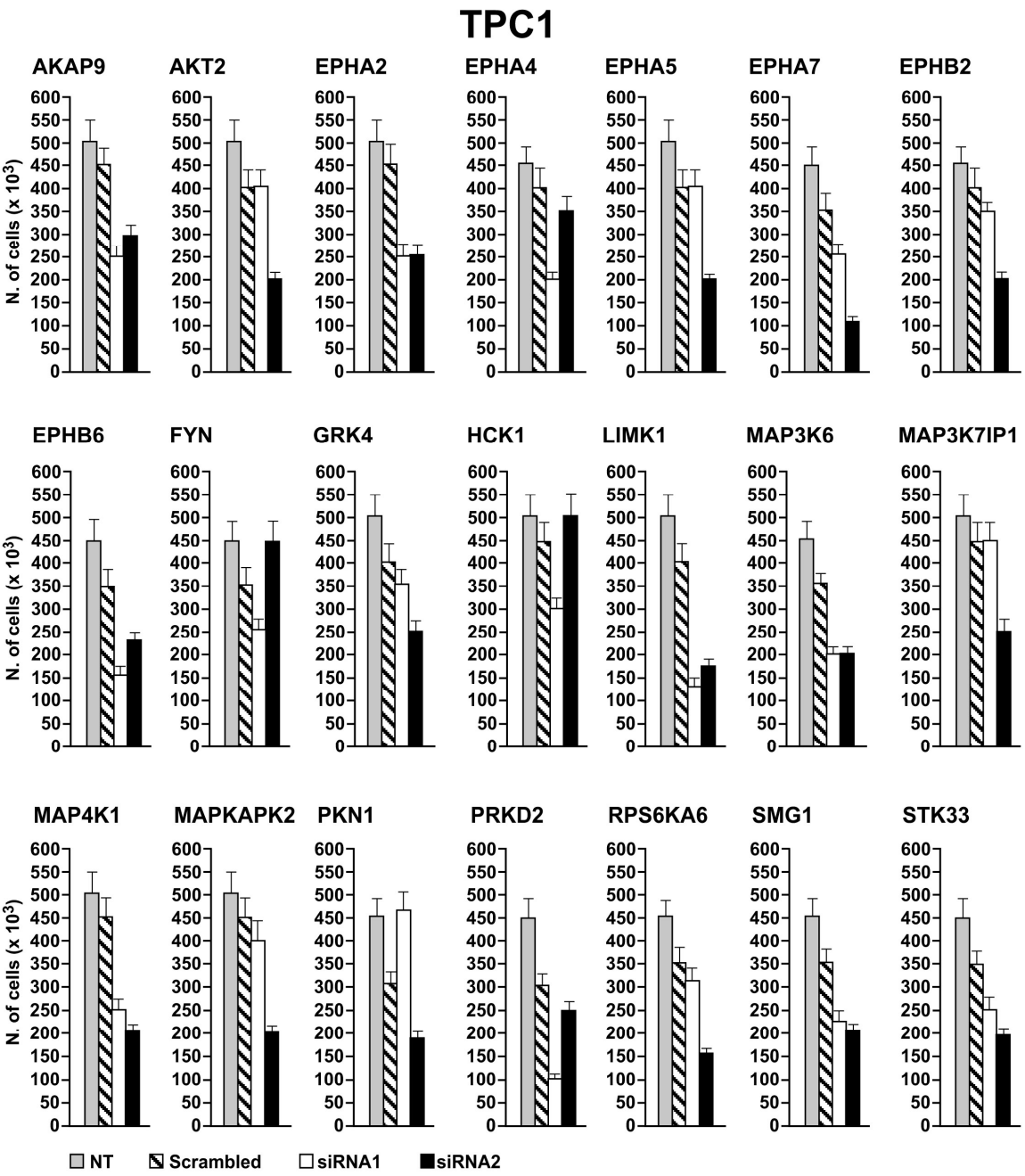

Figure 15: Cell Count of TPC1 cells upon siRNA transfection.

The two different siRNAs against the 21 hits were transiently transfected in triplicate in TPC1 cells. Cells were counted after 72 hours; untransfected and scrambled siRNA transfected cells were used as controls.

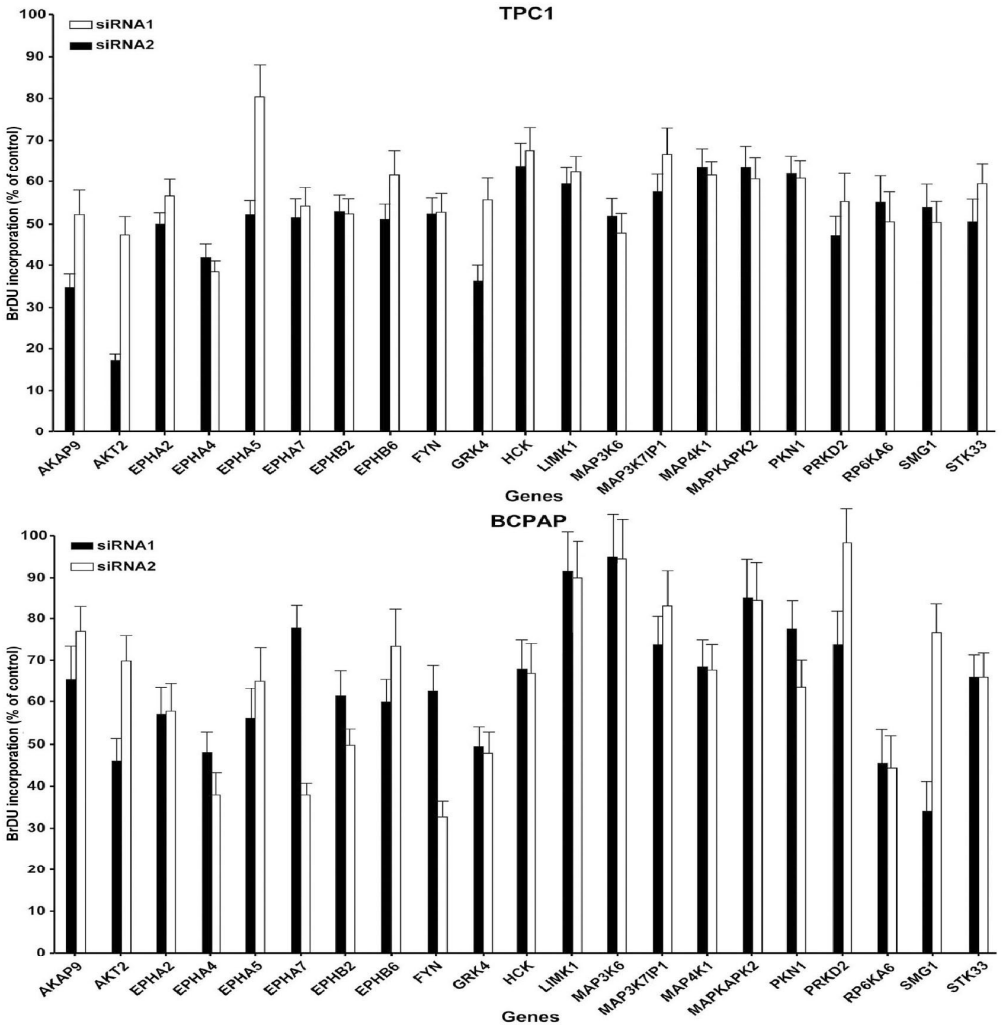

Figure 16: BrdU incorporation measurement upon siRNA transfection.

Cells were transiently transfected with the two siRNAs targeting the 21 hits. After 72 hours, cells incorporating BrdU were counted in triplicate by indirect immunofluorescence. Data are reported as percentage of decrease in BrdU incorporation in comparison to scrambled control.

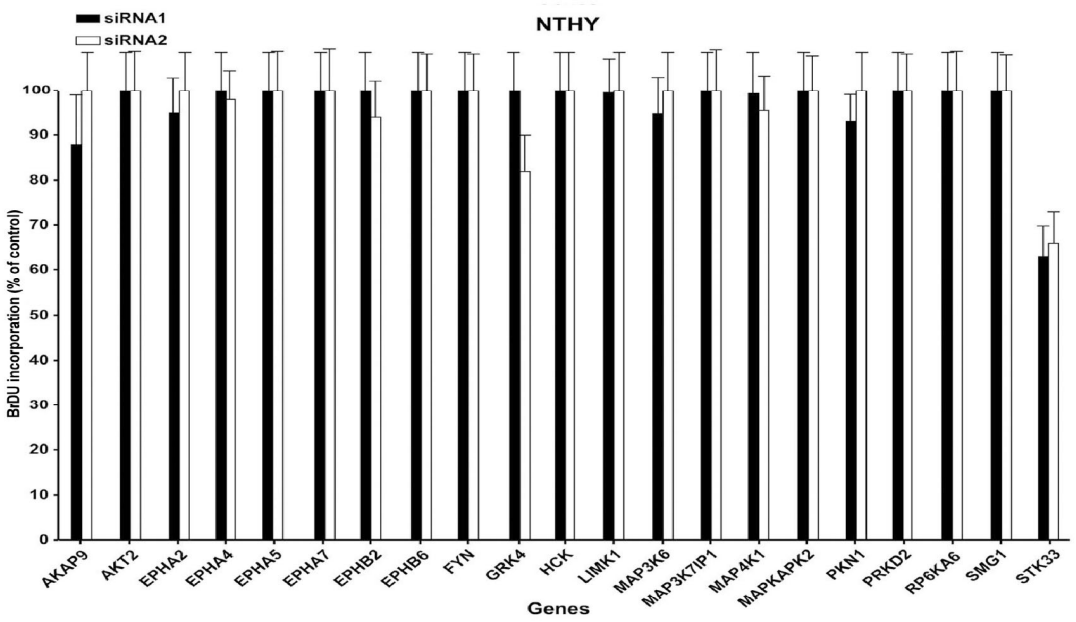

Figure 17: BrdU incorporation measurement upon siRNA transfection.

NTHY cells were transiently transfected with the two siRNAs targeting the 21 hits. After 72 hours, cells incorporating BrdU were counted in triplicate by indirect immunofluorescence. Data are reported as percentage of decrease in BrdU incorporation in comparison to scrambled control.

5.5 Identification of EPH receptor tyrosine kinases as essential mediators of thyroid cancer cell viability.

Among the identified hits, we focused our attention on ephrin receptors (EPH). Six EPH belonging to the A (EPHA2, EPHA4, EPHA5, EPHA7) and B (EPHB2 and EPHB6) families, were among the most prominent hits in our primary screening. EPH receptors bind to either transmembrane (ephrins B for EPHB) or GPI-linked (ephrins A for EPHA) ligands and mediate signaling in a paracrine and autocrine manner (Pasquale, 2010). Recent evidences suggest strong involvement of EPH/EFN in tumorigenesis and correlate their expression levels to invasiveness, metastatization and reduced patient survival rate (Pasquale, 2010).

We initially tested by quantitative RT-PCR whether EPH were differently expressed in cancer comparing to normal thyroid cells. By using a large panel of thyroid cancer cell lines of different histotype and genotype (Table 3), we demonstrated that four (EPHA2, EPHA4, EPHA7 and EPHB2) of the six EPH identified as hits, although with some variability, were over-expressed in most the neoplastic cells comparing to normal P5 cells (Fig. 18).

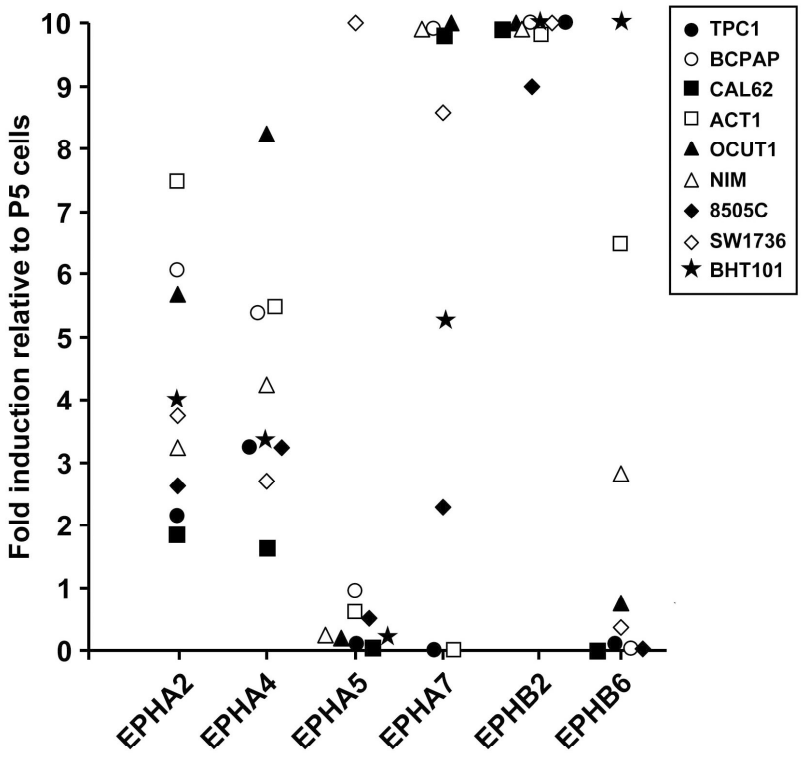

Figure 18: mRNA expression levels of EPH identified as hits in thyroid cancer versus normal cells.

Because EPH are tyrosine kinases, we evaluated their phosphorylation levels in cancer cells as compared to normal cells. To this aim, we used a commercial phospho-tyrosine kinase receptors antibody array that allows to monitor simultaneously the phosphorylation levels of all human RTK. As shown in figure 19, in TPC1, but not in normal NTHY cells, EPHA2, EPHA4 and EPHB2 were among the most prominently phosphorylated spots.

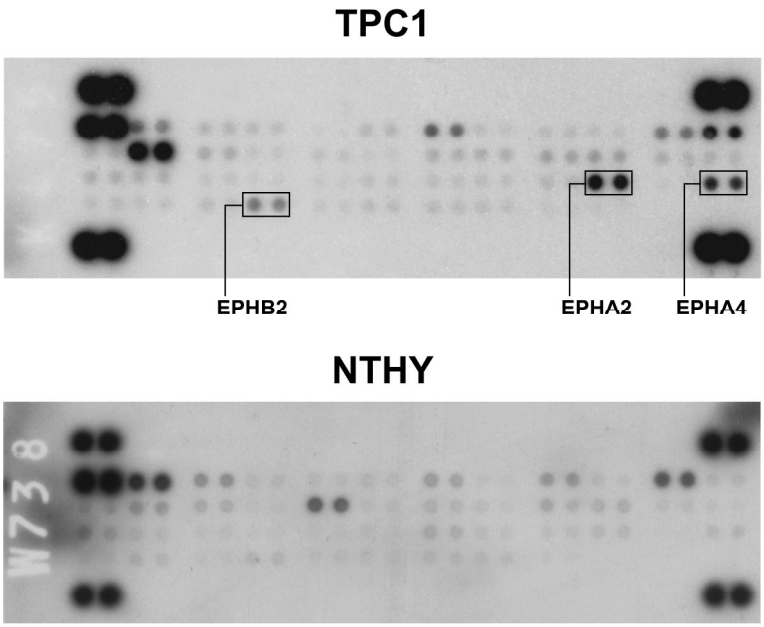

Figure 19: Phosphoantibody array probed with TPC1 and NTHY cell lysates.

5.6 EPHA2, EPHA4 and EPHB2 mediate motile and invasive phenotype of thyroid cancer cells.

We examined EPHA2, EPHA4 and EPHB2 role in cell migration by a wound healing assay. This was done by monitoring cell migration into a “wound” that is created in the cell monolayer in the culture dish. As shown in figure 20, upon silencing of EPHA2, EPHA4 or EPHB2, TPC1 cells showed a greatly impaired ability to close the wound at 12 hours.

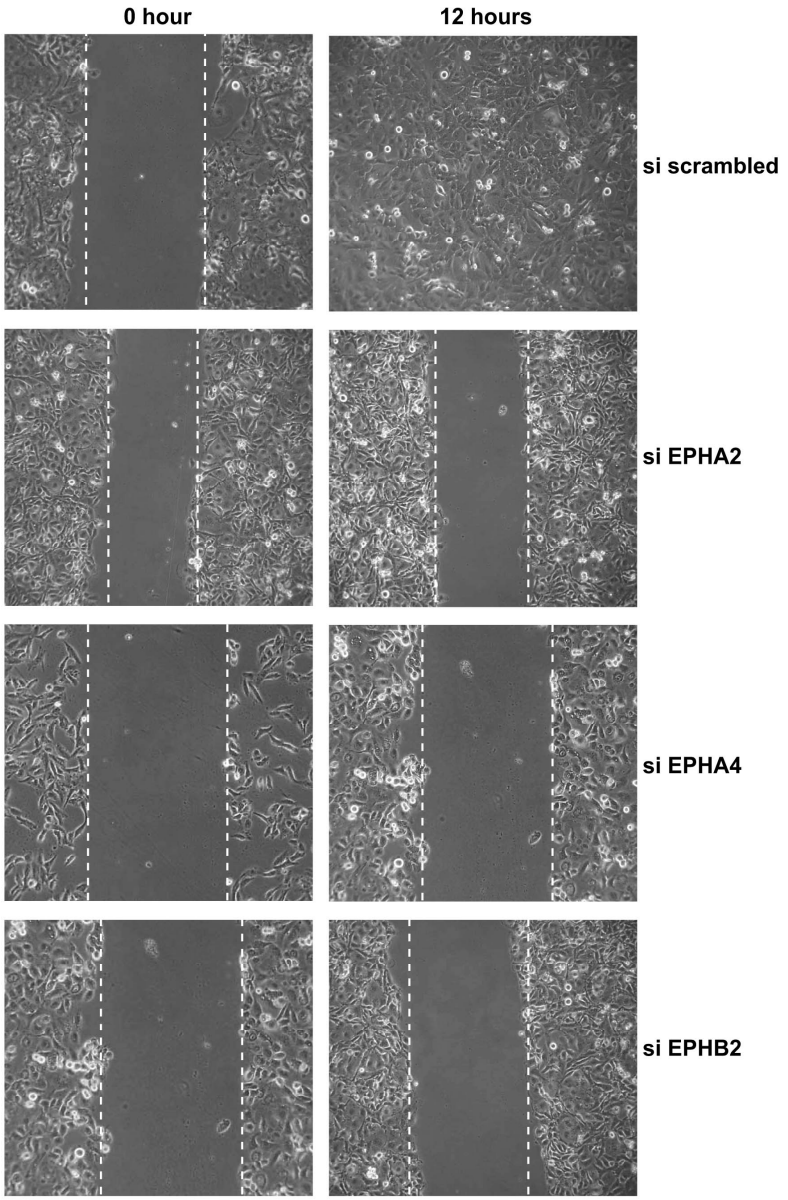

Figure 20: Impaired wound closure in TPC1 cells upon EPH silencing.

Scrambled siRNA and EPH siRNA transfected TPC1 cells were grown in monolayer. A wound was created and cell migration was monitored after 12 hours. The dotted lines define the area lacking cells.

We also examined *in vitro* cell invasion through a Matrigel reconstituted matrix. As shown in figure 21, EPHA2, EPHA4 and EPHB2 siRNAs strongly inhibited TPC1 cell migration into Matrigel.

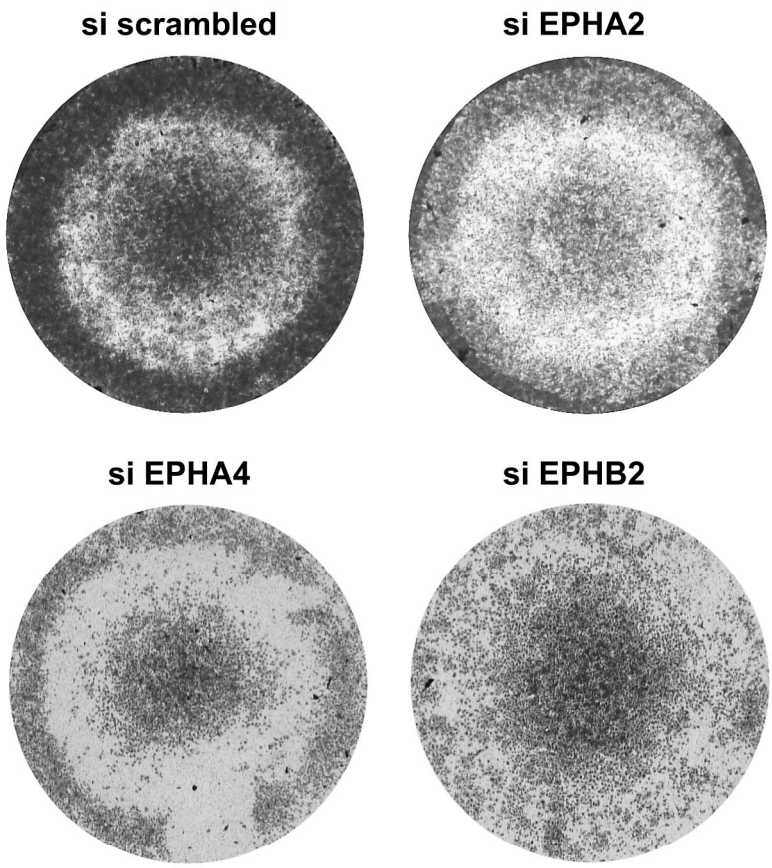

Figure 21: Matrigel invasion of scrambled siRNA and EPH siRNA transfected TPC1 cells.

5.7 EPH expression in human thyroid tumors.

Finally, we investigated by Q-RT-PCR mRNA expression levels of EPH in a small set of thyroid carcinomas of different histotypes. As shown in figure 22, EPHA2, EPHA4 and EPHB2 were over-expressed in PTC and ATC samples with respect to normal thyroid tissues. EPHA2 was the most prominently overexpressed, while EPHA4 and EPHB2 were overexpressed in the majority albeit not all tumor samples.

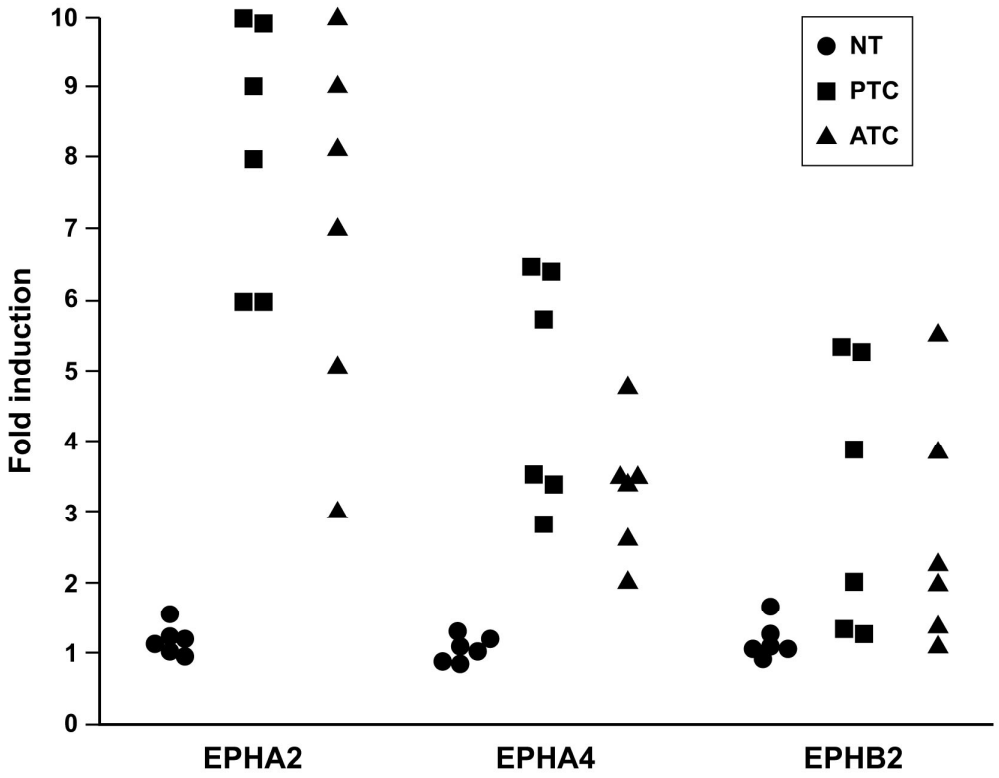

Figure 22: Expression of EPHA2, EPHA4 and EPHB2 in thyroid tumor samples.

RNA was extracted from 6 normal thyroids, 6 PTC and 6 ATC samples and subjected to a Q-RT-PCR in triplicate to check the expression of the indicated EPH. Results are reported as fold change with respect to the average expression levels of the normal tissues.

6. DISCUSSION

6.1 Results of the screening.

Elucidation of the complex signaling pathways governing cancer cell growth and survival has allowed the rational design of targeted inhibitors. Still, a major challenge is that of determining which target to inhibit in each cancer type. Most of the genetic lesions causing the various types of thyroid cancer are known. PTC is associated to RET and BRAF gain, FTC to RAS, MTC to RET and PDC and ATC to RAS, BRAF, CTNNB1 and PI3K (Kondo *et al*., 2006). All these lesions are susceptible of pharmacological intervention and this makes thyroid cancer a good model whereby to exploit molecularly targeted cancer treatments (Santoro *et al*., 2006; Sherman, 2009). However, while RET and BRAF inhibitors are in advanced clinical experimentation (Wells *et al*., 2009; Pratilas *et al*., 2010) and many PI3K/AKT inhibitors are progressing rapidly into the clinics (Engelman *et al*., 2007), targeting other oncoproteins like RAS proved to be a very difficult task (Bommi-Reddy *et al*., 2010).

Cancer cells can be addicted to the genetic lesions that have initiated the transformation process ("oncogene addiction") (Luo *et al*., 2009). Examples are BCR-ABL in CML (Sawyers, 2010), EGFR in NSCLC (*n*on-*s*mall-*c*ell *l*ung *c*ancer) (Ciardiello *et al*., 2008), KIT in GIST (Antonescu, 2010) and BRAF in melanoma (Vultur *et al*., 2010). However, cancer cells can also be addicted to proteins that, though non mutated *per se*, still are crucial for cancer (but not normal) cell viability.

This phenomenon has been called “non-oncogene addiction” (Luo *et al*., 2009). Thus, as an example, RAS mutated cancer cells can in principle be eradicated by identifying proteins (other than RAS) to which they are addicted to. The identification of such Achilles’ heels remains a formidable challenge. Moreover, also in those cases in which cancer-causing oncoprotein can be effectively targeted (for example BCR-ABL in CML), resistance may develop. In some cases, the resistance is mediated by secondary mutations in the oncoprotein that impair drug binding; in other cases, resistance is caused by the activation of alternative pathways with which cancer cells escape the treatment (Gramza *et al*., 2009; Milojkovic *et al*., 2009).

RNAi technology has provided a powerful approach to tackle these tasks, because it allows to search for proteins to which cancer cells are addicted to or that mediate cancer cell resistance (Luo *et al*., 2009). In this Dissertation, we applied a RNAi-based screen to identify protein kinases to which thyroid cancer cells are addicted to. We focused on protein kinases (and associated proteins) because of their frequent involvement in human cancer (in thyroid cancer in particular) and because of their “druggability”. Initially, we conducted the screening in the TPC1 cell line, expressing the RET/PTC1 rearrangement. Then, we extended the study to other thyroid cancer cell lines bearing BRAF or RAS oncogenes. At the end, we have identified a set of 21 genes that, when silenced, impaired the viability of the various thyroid cancer cells. Importantly, sensitivity to knock-down of these 21 genes was not cell line-specific and, therefore, not oncogene-specific (RET/PTC, BRAF, RAS). Indeed, on average, all the 4 cancer cell lines were equally susceptible to their blockade. Exceptions were LIMK1 and MAP3K, that were important for TPC1 cells only, and EPHA5, HCK, MAP3K7IP1, PKN1 and PRKD2 that were required for the PTC (TPC1 and BCPAP) cells only.

These findings anticipate that BRAF mutant thyroid cancers may be susceptible to the inhibition of most of our 21 hits, an important concept because BRAF mutations are recognized as risk factors for thyroid cancer to progress to radioiodine refractory disease (Xing, 2007). Moreover, inhibition of most of our 21 genes may exert efficacy also in RAS mutant thyroid cancers; this is also important because, as mentioned above, RAS oncoproteins have been difficult to be targeted direclty. Three out of the 4 cancer cell lines used bear p53 mutations. Therefore, efficacy of our siRNAs was not negatively affected by p53 mutations; this is another important point given the role that p53 mutation exterts on cancer cell resistance to pharmacological therapy (Wiman, 2010). Finally, the two non tumorigenic thyroid cell types were in general refractory to the effects of the identified siRNAs, a fact that warrants a good therapeutic window for approaches aimed at inhibiting them in thyroid cancer. Blockade of the 21 hits quite homogenously affected cell proliferation but not cell survival (STK33 silencing caused also apoptosis). This suggests that they are primarily involved in sustaining proliferation. However, this does not exclude that specific hits may also exert additional functions. In fact, EPH receptors, for instance, were important also for cancer cell migration and invasion.

6.2 Signaling pathways and networks involved in thyroid cancer cells proliferation.

Table 5 summarizes the most important signaling features of the 21 hits. They included: 2 cytosolic tyrosine kinases; 6 receptor tyrosine kinases (all belonging to the EPH family); 11 serine/threonine kinases; 2 kinase-interacting proteins. None of them has been previously direclty involved in thyroid cancer; exceptions were AKAP9 that was found rearranged with BRAF in radiation-associated PTC (Ciampi *et al.,* 2005) and GRK4 that was found overexpressed in thyroid nodules (Voigt *et al.,* 2004). Most of our 21 hits are involved in cell cycle control. Some of them are also involved in promoting metastasis (AKT2, LIMK1, MAP3K7IP1), tumor angiogenesis (MAP3K6, PRKD2), mediating resistance to tyrosine-kinase inhibitors (FYN, HCK) (see Table 5 and references herein).

Table 5: Features of the 21 hits identified
(ser/thr kinases are highlighted in yellow; kinase-interacting proteins are in green; receptor and non receptor tyrosine kinases are in blue or gray, respectively)

	Gene	Function	Features	Cancer correlations
1	**AKAP9**	A kinase (PRKA) anchor protein 9: Kinase-interacting protein	Belongs to a group of structurally diverse proteins which have the common function of binding to the regulatory subunit of protein kinase A (PKA) and confining the holoenzyme to discrete locations within the cell.	• Variants associated to breast cancer risk (Frank 2008) • Rearranged with BRAF in radiation-associated PTC (Ciampi 2005)
2	**AKT2**	Serine/threonine kinase	One of the 3 Akts: Akt1, Akt2, and Akt3 (Fig. 22)	• Amplified in ovarian and pacreatic carcinomas (Chen 1992, 1996) • Involved in breast and colorectal cancer metastasis (Wickenden 2010; Rychahou, 2008)
3	**EPHA2**	RTK	See text for details	
4	**EPHA4**	RTK		
5	**EPHA5**	RTK		
6	**EPHA7**	RTK		
7	**EPHAB2**	RTK		
8	**EPHB6**	RTK		
9	**FYN**	Tyrosine kinase	SRC-like tyrosine kinase	• Involved in EGFR and BCR-ABL mediated transformation (Lu, 2009; Ban, 2008) • Involved in CML drug resistance (Grosso, 2009)

11	**HCK**	Tyrosine kinase	Src family tyrosine kinases	• Involved in BCR-ABL signaling (Meyn, 2006) • Mediates CML resistance to ABL inhibitors (Pene-Dumitrescu, 2010)
12	**LIMK1**	LIM domain containing ser/thr kinase	regulator of actin cytoskeleton remodeling	• Required for path generation by tumor cells during collective tumor cell invasion; mediates matrix protein degradation and invadopodia formation (Scott, 2010) • Involved in metastasis (Vlecken, 2009) • Activated by GDNFRa (Yoong, 2009)
13	**MAP3K6**	mitogen-activated protein kinase kinase kinase 6. Also named ASK2	Ser/thr kinase and activator of c-Jun kinase (MAPK7/JNK) and p38 MAPK	• Sustains tumor cell proliferation and VEGF secretion (Eto, 2009)
14	**MAP3K7IP1**	TGF-beta activated kinase 1/MAP3K7 binding protein 1	Regulator of the MAP kinase kinase kinase MAP3K7/TAK1, which mediates TGF beta, interleukin 1, and WNT-1 pathways. This protein can also activate the mitogen-activated protein kinase 14 (MAPK14/p38alpha) (Fig. 23)	• Its interactor TAK1, is involved in pro-metastatic activity of TGFb in breast cancer (Neil, 2008)
15	**MAPKAPK2**	Ser/Thr protein kinase. Also named MK2	This kinase is regulated through direct phosphorylation by p38 MAP kinase.	• Involved in cell cycle checkpoint in p53 defective cells (Rheinardt, 2010)
16	**MAP4K1**	mitogen-activated protein kinase kinase kinase kinase 1	Involved in NFkB and JNK activation	• Suppressed in pancreatic carcinoma (Wang, 2009) • Sustains tumor growth in colon cancer (Yang, 2006)
17	**PKN1**	protease-activated ser/thr kinase 1	Activated by Rho family of small G proteins and by PDK1. Subject to proteolytic activation by caspase-3	• Overexpressed in ovarian carcinomas (Galgano, 2009) • Activates AR in prostate cancer (Metzger, 2003)
18	**PRKD2**	ser/thr-protein kinase D2	Activated by phorbol esters. It can bind to diacylglycerol in the trans-Golgi network (TGN) and regulate basolateral membrane protein exit from TGN.	• Involved in VEGF secretion by pancreatic and gastric cancers (Azoitei, 2010)
19	**RPS6KA6**	ribosomal protein S6 ser/thr kinase (RSK4)	Substrate of ERK, promotes cell survival though the inactivation of several apoptotic effectors, and mediates cell growth and proliferation (Fig. 23)	• RSK family members are upregulated in many cancer types (Carriere, 2008) • RSK4 however is often downregulated and favours cell senescence (Lopez-Vicente 2009; Thakur, 2008)
20	**SMG1**	SMG1 homolog, phosphatidylinositol 3-kinase-related ser/thr kinase	involved in nonsense-mediated mRNA decay (NMD) as part of the mRNA surveillance complex.	• Negatively regulates HIF (Chen, 2009) • Mediates response to genotoxic stress (Brumbaugh, 2004) • Has anti-apoptotic activity (Oliveira, 2008)
21	**STK33**	ser/thr kinase 33	Involved in positive regulation of S6K downstream substrate of mTOR, the target of rapamycin (Fig. 22)	• STK33 is required in *K-RAS*–mutant cells to probably a normal cellular protein, the function of which *K-RAS*–mutant cells rely on. This phenomenon has recently been termed "non–oncogene addiction" (Downward, 2009; Scholl, 2009)

Table 5: Features of the 21 hits identified.

Ser/thr kinases are highlighted in yellow; kinase-interacting proteins are in green; receptor and non-receptor tyrosine kinases are in blue and gray, respectively.

Although our hits are involved in several pathways and cellular functions, the list was enriched for: i) EPH (discussed below); ii) SRC family kinases (FYN, HCK); iii) proteins involved in the p38 MAPK pathway (MAP3K6, MAP3K7IP1, MAPKAPK2) or other MAPK cascades (RPS6KA6 in the ERK; MAP3K6 in the JNK); iv) proteins involved in the PI3K/mTOR signaling (AKT2, STK33) (Table 5). The PI3K/mTOR and MAPK pathways are depicted in figures 23 and 24.

Involvement of these cascades in thyroid cancer is well established. Moreover, both PI3K and ERK are key effectors of RET-derived oncogenes (Melillo *et al*., 2005; Lodyga *et al*., 2009). Also SRC family kinases were previously involved in RET/PTC signal transduction (Melillo *et al*., 1999) and as targets for kinase inhibitors in thyroid cancer (Schweppe *et al*., 2009). Finally, JNK and p38MAPK were activated by RET/PTC (Chiariello *et al*., 1998) and p38 targeting was effective in reducing proliferation of TPC1 cells (Mariggio *et al*., 2007).

The function of STK33 serine/threonine kinase is still quite obscure; probably, it is involved in S6K activation downstream mTOR (Fig. 23). Noteworthy, STK33 was identified as one of the proteins to which RAS-transformed cells are addicted and, therefore, proposed as a potential drug target to combat RAS positive human cancers (Downward, 2009; Scholl *et al*., 2009). Although in our screen STK33 silencing affected both normal and tumoral thyroid cells, its effects in terms of cell viability were greater in TPC1 and BCPAP cells. Thus, the possibility that also in the case of thyroid cancer, tumor cells might be more susceptible to STK33 inhibition than normal cells should be investigated in greater details.

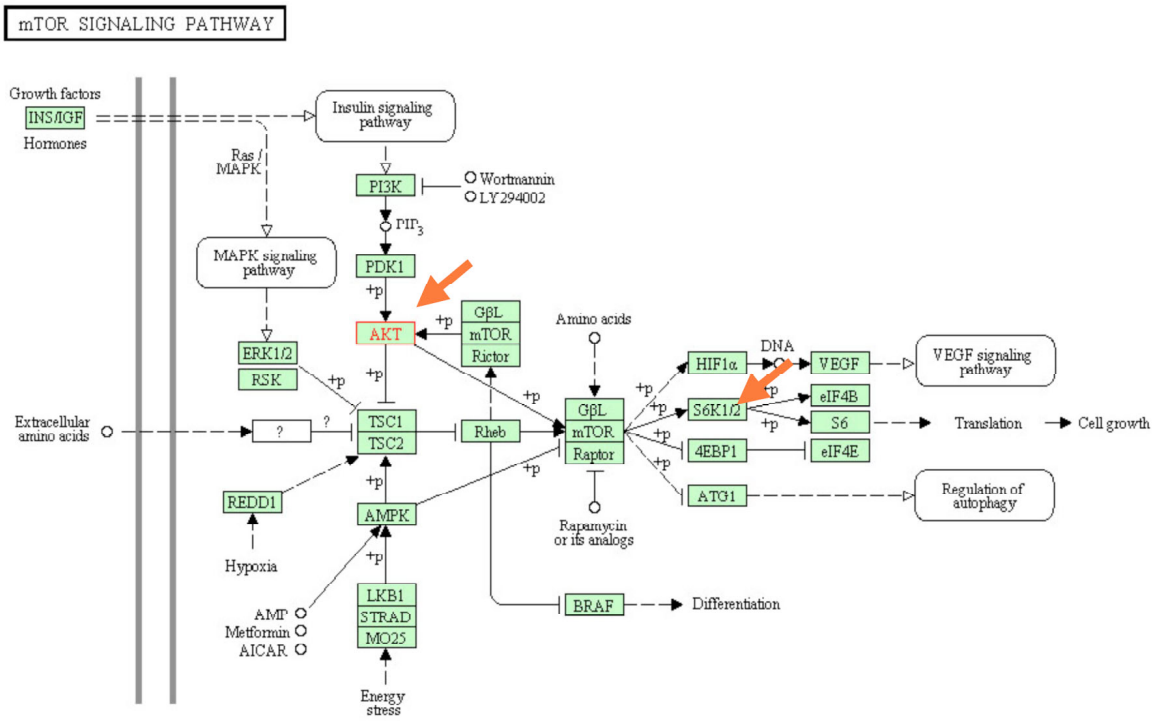

Figure 23: The mTOR signaling pathway.

Red arrows indicate the level of action of AKT2 and STK33.

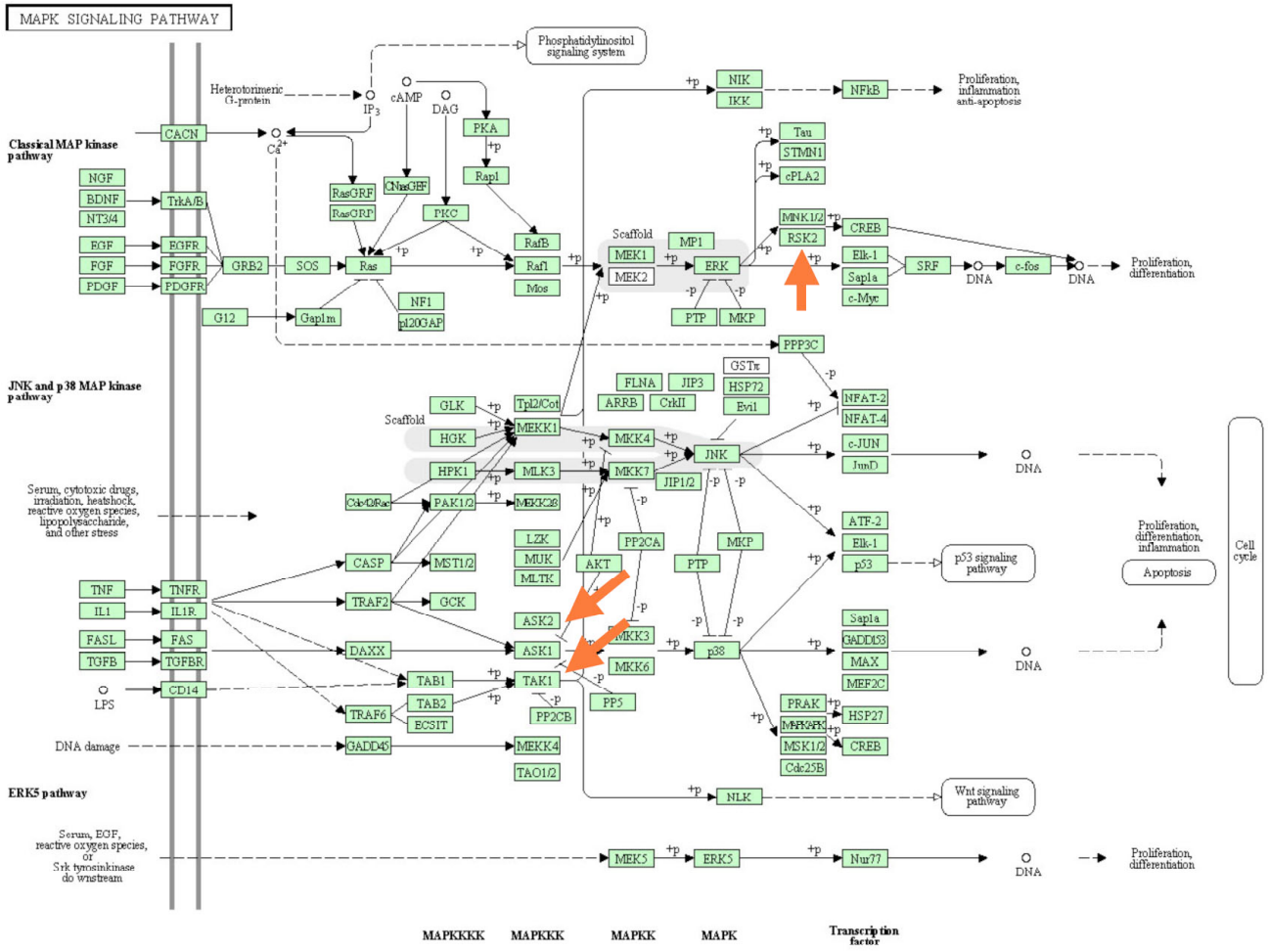

Figure 24: The MAPK (JNK, p38MAPK and ERK) signaling pathways.

Red arrows indicate the level of action of MAP3K6 (ASK2), RPS6KA6 (RSK) and MAP3K7IP1.

6.3 EPH in thyroid cancer.

RTK receptors of the EPH family were the largest group (6/21, 28%) among the hits identified. Indeed, four members of the A family (EPHA2, EPHA4, EPHA5, EPHA7) and two of the B family (EPHB2 and EPHB6), were included among them. All of them (but not EPHA5, that was required for TPC1 and BCPAP only) were required for the viability of all thyroid cancer cells. Four of them (EPHA2, EPHA4, EPHA7 and EPHB2) were over-expressed in most the neoplastic cell lines as compared to normal P5 cells; three of them (EPHA2, EPHA4 and EPHB2) were constitutively phosphorylated in TPC1 but not in NTHY cells. Knock-down of EPHA2, EPHA4 and EPHB2 not only reduced proliferation but also cell motility and invasiveness. Finally, EPHA2, and to a lower extent EPHA4 and EPHB2, were over-expressed in PTC and ATC comparing to normal thyroid specimens. Possibly, EPHA5 and EPHB6, that were required for TPC1 viability but were not over-expressed in TPC1, are involved in TPC1 with different mechanisms either than upregulation. Both of them, however, were upregulated at least in one thyroid cancer cell line other than TPC1 (Fig. 18). EPHA7, that was essential for TPC1 cell viability, was upregulated in all thyroid cancer cell lines, but not in TPC1; it is probably for this reason that EPHA7 was not detected in our TPC1 phosphoarray immunoblot (Fig. 19). In this study we focused on EPHA2, EPHA4 and EPHB2 because they fullfilled all criteria (viability requirement, overexpression, constitutive phosphorylation), but all the other identified EPH will require further investigation.

Mechanism of overexpression and activation of EPH in thyroid cancer cells remain to be addressed. Intriguingly, EPH expression is stimulated by the RAS-RAF-ERK axis (Macrai *et al*., 2005); being virtually always activated in thyroid cancer (through RET, RAS or BRAF), this pathway may explain EPH overexpression in thyroid cancer cells.

EPHB receptors bind promiscuously to the 3 transmembrane ephrins B (EFN-B), while EPHA receptors bind promiscuously to the 5 GPI-linked ephrins A (EFN-A). EPHA4 can also bind EFN-B and EPHB2 can also bind EFN-A5. A major ligand for EPHA2 is EFNA1 (Pasquale, 2010). EFNs are typically expressed in microenviroment (endothelial cells and pericytes) but can also be expressed in tumor epithelial cells. Since in TPC1 cells EPHA2, EPHA4 and EPHB2 were constitutively phosphorylated, it can be anticipated that some EFNAs and EFNBs are expressed in TPC1 cells. Possibilities include EFNA1 for EPHA2, any EFNB for EPHB2 and EFNA5 for EPHB2. The search for the specific EFNs involved in thyroid cancer will require further investigation. It will be also important to study the expression of EPH-EFN interaction in thyroid tumor vessels endothelial cells, because EPH are able to promote tumor angiogenesis (Pasquale, 2010).

EPH-EFN complexes emanate bidirectional signaling: forward signals that depend on EPH tyrosine-kinase activity, and reverse signals depending on SRC family kinases associated to the cytosolic side of EFN (Pasquale, 2010). Forward signaling controls many functions including cell migration, invasion, proliferation ad survival.

Among them, perhaps, the most typical EPH-mediated effect is the generation of a "repulsive" inter-cellular force that leads to cell-cell detachment and scattering (Pasquale, 2005). Importantly, such a migration-promoting role was exerted by EPHA2, EPHA4 and EPHB2 in thyroid cancer cells (Figs. 20, 21).

EPHA1 was initially isolated as an oncogene able to transform NIH3T3 fibroblasts (Hirai *et al*., 1987; Maru *et al*., 1990). EPH (mainly EPHA2 and EPHB4) and EFN over-expression was correlated with cancer progression and metastatic spread in breast and prostate carcinomas (Wykosky *et al*., 2008; Zhuang *et al*., 2010; Kumar *et al*, 2009; Huang *et al*., 2007). These findings are in line with our data suggesting an oncogenic role of EPH in thyroid cancer. However, EPH function is characterized by puzzling dichotomies and many findings claiming a tumor suppressor rather than oncogenic role of EPH have been reported. Indeed, EPHA1 and EPHB are down-regulated in advanced colorectal carcinomas (Alazzouzi *et al*., 2005; Herath *et al*., 2009), EPHB6 is down-regulated in metastatic lung cancer (Yu *et al*., 2010), some EPH receptors map in chromosomal regions that are often deleted in human cancer (1p36), and, finally, EPH mutations found in prostate, gastric, lung and colorectal tumors can impair kinase function (Ding *et al*., 2008) (see also Table 6). To support this putative tumor suppressor role, forward EPH signaling has been shown to suppress oncogenic signaling, blocking PI3K and RAS pathways (Pasquale, 2010). For instance, EPH signaling may activate p120GAP (thus blocking RAS), SHP2 phosphatase (thus blocking AKT) and TSC complex (thus blocking mTOR) (Pasquale, 2010). In this way, the physiological function of EPH might be that of preserving epithelial cell fate and promote contact-mediated inhibition (Pasquale, 2005).

One possibility for cancer cells to overcome this tumor suppressor role is to prevent EPH forward signaling by lowering EFN expression (Batlle *et al*., 2005; Macrae *et al*., 2005). Moreover, recent observations suggest that the "tumor-suppressor" roles attributed to EPH can be subverted to an "oncogenic" one in cancer cells. In some cell contexts, EPH forward signaling activates oncogenic, like AKT, ABL, RHO and RAC pathways, rather than tumor suppressor ones (Pasquale, 2010). Finally, in some cases, EPH signaling is subverted from a suppressor to an oncogenic role because of the simultaneous activation of other pathways in cancer cells. For instance, association to ErbB2 or phosphorylation mediated by AKT enable EPHA2 to activate rather than inhibit RAS-ERK (Brantley-Sieders *et al*., 2008; Miao *et al*., 2009). In thyroid cells, EPHA2, EPHA4 and EPHB2 are overexpressed and constitutively phosphorylated; moreover, their knock-down impair cell proliferation and inhibits migration and invasiveness. Therefore, these EPHs behave as *bona fide* oncogenes. However, whether these effects are opposite to those that EPH may mediate in normal thyrocytes and the biochemical mechanism for such a subversion need to be further investigated.

6.4 Possible mutations of the 21 hits.

Recent systematic genome sequencing efforts have searched for novel oncogenic mutations in cancer samples (Sjoblom *et al*. 2006; Weir *et al*. 2007; Wood *et al*., 2007; Jones *et al*., 2008; Ding *et al*. 2008; Kan *et al*, 2010). Some of these mutations are catalogued in COSMIC (catalogue of somatic mutations in cancer database) (Tab. 6).

	Gene	N° of Mutations in Ding (2008)	N° Mutations in Wood (2007)	N° Mutations in Kan (2010)	N° Mutations in COSMIC
1	AKAP9	0	3	10	7
2	AKT2	0	0	1	0
3	EPHA2	1	0	0	4
4	EPHA4	1	1	2	8
5	EPHA5	7	0	5	13
6	EPHA7	5	1	2	13
7	EPHB2	0	0	0	1
8	EPHB6	5	4	1	12
9	FYN	2	0	1	5
10	GRK4	0	0	0	1
11	HCK	4	0	0	6
12	LIMK1	0	0	0	1
13	MAP3K6	2	1	0	7
14	MAP3K7IP1	0	0	1	0
15	MAP4K1	3	0	1	5
16	MAPKAPK2	0	0	0	0
17	PKN1	0	1	0	4
18	PRKD2	0	0	0	5
19	RPS6KA6	2	0	0	4
20	SMG1	3	0	0	10
21	STK33	0	0	0	3

Table 6: List of down hits and mutations found in previous studies.

By comparing our 21 hits to cancer genome-sequencing papers, we found that virtually all of them had been identified as bearing somatic mutations in tumor tissues or tumor-derived cell lines (Tab. 6). Though the functional meaning of these mutations is still unknown in the majority of the cases, this reinforces the concept that our hits may play a role in thyroid cancer and paves the way to studies aimed at searching for their mutations in thyroid cancer samples.

6.5 Future perspectives.

With this study we have searched for novel kinases involved in thyroid cancer. The screening highlighted some specific pathways (PI3K, ERK, JNK, p38) and showed that EPH may play a particularly important role in thyroid cancer. Several points remain to be addressed, including:

- mechanism with which EPH gain can contribute to thyroid cell transformation (e.g. role of the EFN ligands);
- mechanism with which (e.g. overexpression or increased activity) the non-EPH hits could be involved in regulating thyroid tumorigenesis;
- presence of somatic mutations in any of the 21 hits;
- *In vivo* role of the identified hits (e.g. in animal models);
- possibility of pharmacological targeting alone and/or in combination with RET, BRAF, PI3K inhibitors.

Nothwithstanding these limitations, we can conclude that with study we could identify a set of novel genes that play a key role in thyroid cancer cells viability and that EPH, in particular, are novel RTK involved in thyroid cancer.

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Attached manuscript # II

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**These two authors equally contributed to the study*

Sonic Hedgehog pathway is upregulated in thyroid cancer.

Manuscript in preparation

Sonic Hedgehog pathway is upregulated in thyroid cancer

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ABSTRACT

Dysregulation of Sonic Hedgehog (Shh) signaling pathway has been implicated in many familial and sporadic cancers. In this study, normal and neoplastic thyroid samples and thyroid cell lines have been examined for the expression of the Shh signaling pathway components. Expression of the Shh ligand (Sonic), its receptors Patched (Ptch) and Smoothened (Smo), and the Shh final effector, the trascription factor Gli1, was higher in benign and malignant thyroid tumors than in normal cells. Moreover, Gli1-Luciferase reporter was active in thyroid cancer cells and cyclopamine, an inhibitor of Smo, blunted thyroid cancer cell viability. In conclusion, Shh is a novel pathway and a potential therapeutic target in thyroid cancer.

INTRODUCTION

Thyroid cancer accounts for 2.6% of all new cancers, when epithelial skin cancers are excluded (6). The molecular basis of thyroid carcinogenesis has been widely investigated, and oncogenes such as BRAF, RAS and RET have been identified as major players in tumor development and aggressiveness (2). This dependence on specific genetic lesions has highlighted thyroid cancer as an ideal target for molecularly targeted new therapeutics (15). Identification of new mediators of thyroid carcinogenesis will be crucial to tackle the development of new therapeutics for thyroid cancer, particularly to treat those patients that are negative for the known mutations and those that become resistant to molecular inhibitors.

The vertebrate Hedgehog family is represented by three members: Desert (Dhh), Indian (Ihh) and Sonic (Shh) Hedgehog. Sonic (Shh) is the best-characterized family member and it is involved in a wide variety of developmental events. In humans, Shh gene is located on chromosome 7 (7q36) and its heterozygous mutation results in holoprosencephaly (13). Shh acts both as a short-range and a long-range diffusible morphogen and regulates epithelial-mesenchymal interactions in many organs. Shh signals are transduced, at the cell surface, by two transmembrane proteins, Patched (Ptc) and Smoothened (Smo). Smo is a 7-transmembrane domain receptor required to transduce the Shh signal. Ptc is a 12-pass membrane protein that binds directly to Shh ligands and inhibits Smo. Shh binding suppresses Ptc inhibitory activity; thus, Shh binding to Ptc phenocopies the genetic loss of Ptc. In the absence of Ptc, Smo constitutively activates a still poorly known molecular events that result in the accumulation and nuclear translocation of Gli transcription factors, resulting in the activation of Gli targetd genes (3, 9).

In normal tissues, Shh pathway plays a key role in embryogenesis as well as in the maintenance of stem cell compartments during adulthood. Shh has recently been described also as a regulator of thyroid development (6), because it governs symmetric bilobation of the thyroid gland and represses inappropriate thyroid differentiation in embryonic tissues outside the thyroglossal duct. Genetic deletion of Shh caused hemiagenesis and ectopic development of the thyroid gland in a mouse model (6). Abnormal activation of the Shh pathway, because of gene mutations or overexpression, has been reported in many cancers, including basal cell carcinoma, medulloblastoma, pancreatic, breast, lung, prostate and ovarian cancers (20).

Here, we have investigated the presence of the Shh pathway components in a panel of thyroid cancer cells and human tumor samples. Our results show an increased expression of Shh, Ptc, Smo and Gli1 in thyroid tumors as compared to normal control. In cell lines, the expression correlated with an increased transcriptional activity of Gli1 that was blunted by cyclopamine, an inhibitor of Smo. Finally, cyclopamine treatment impaired proliferation and survival of thyroid cancer cells.

RESULTS

Shh pathway components are expressed in thyroid tumors.

Tumors of the thyroid gland include benign adenomas, differentiated -papillary (PTC) and follicular (FTC)- and undifferentiated (anaplastic: ATC) thyroid carcinomas. We analyzed the expression of Gli1 by immunohistochemistry in a panel of 40 thyroid tumors of different histotypes (10 for each group). Normal thyroids (n.10) were negative, while tumors were constantly positive, with a proportion of positive cells higher in PTC and ATC than in adenomas and FTC (Table 1, and Figure 1). Among PTC, PTC-TCV (tall-cell variant) positivity was particularly high and PTC-FV (follicular variant) was particularly low. No correlation with tumor size or age of the patients was noted; the percentage of Gli1 positive cells was higher in males than females (63% vs 48%).

We performed quantitative RT-PCR to measure mRNA expression levels of Shh, Gli1 and Ptc in normal, PTC and ATC tissues. As shown in figure 2, all the components of the Shh pathway, though with significant variability among the samples, were expressed in tumors at higher levels than in normal controls; ATC showed the highest expression values of the Shh ligand (Fig. 2).

Shh pathway is active in thyroid cancer cell lines.

We studied the expression of components of the Shh pathway in thyroid cancer cell lines. As shown in figure 3A, Shh ligand expression levels were significantly higher in 3 out of the 6 cancer cell lines with respect to non tumoral NTHY cells. Smo levels were slightly higher in cancer with respect to NTHY cells. Finally, no significant difference between most cancer and normal cells was detected for Ptc and Gli1 (Fig. 3A). These findings supported an autocrine (increased levels of expression of the ligand) mode of activation of the Shh pathway in the majority of thyroid cancer cell lines. An alternative mode of activation might be present in BCPAP cells that lost the expression of the Ptc receptor (Fig. 3A).

Activity of the Gli1 transcription factor is a reliable marker of Shh pathway activity. Thus, we transfected the various cell lines with Gli-Luc promoter. Figure 3B demonstrates that PTC (TPC1 and BCPAP) and ATC (8505C and HTH74) cells had higher levels of luciferase activity driven by Gli1 reporter than non tumoral NTHY cells. This activity was only slightly stimulated by exogenous constitutively active Gli1 transfection. NTHY cells had low levels of luciferase activity that were however strongly increased when exogenous Gli1 was transfected (Fig. 3B).

Cyclopamine is a lipophilic compound extracted from the *Lily Veratrum Californicum*. It antagonizes Smo activity by binding to its heptahelical bundle. Thus, it blocks Shh pathway activation either resulting from Smo or Ptc mutations or from Shh ligand over-expression. Cyclopamine is currently being investigated as a treatment for basal cell carcinoma, medulloblastoma, rhabdomyosarcoma and many other cancer types (11). In thyroid cancer cell lines, Gli reporter activation was Smo-dependent, because cyclopamine treatment completely abolished the luciferase activity (Fig. 3B).

Finally, the increased transcriptional activity of the Gli-Luc reporter was paralled by an increased nuclear accumulation of Gli1 in TPC1 cells as seen by indirect immunofluorescence (Fig. 3C). Cyclopamine treatment reduced both the total signal as well as the nuclear accumulation of Gli1 (Fig. 3C).

Cyclopamine treatment impairs proliferation and survival of thyroid cancer cells.

The various cell lines were exposed to 10 or 20 μM cyclopamine to test their viability and proliferation. As shown in Figure 4A, drug treatment at 10 and 20 μM reduced number of viable cancer cells, while it had only a minor effect on non tumoral cells at 20 μM (Fig. 4A). The reduced viability was paralleled by a modest reduction of proliferative ability of the cells, as shown by reduction of BrdU incorporation in Figure 4B. Importantly, cyclopamine (10 μM) had a strong cytotoxic effect in cancer but not NTHY cells (Fig. 4C). In NTHY a modest cytotoxic effect was detected only at 20 μM dose.

DISCUSSION

Inappropriate activation of Shh pathway leads to malignancy. Several studies have shown that components of the Shh pathway are expressed at high levels in cancer, particularly in aggressive and undifferentiated cancer types (4). Here we have investigated expression and activity of this pathway in thyroid cancer. Our data show an activation of the pathway in thyroid tumors, and particularly in aggressive PTC variants (PTC-TCV) and ATC. Although the mechanism of Shh pathway activation in thyroid tumors will require further studies, our data support a model whereby Sonic ligand upregulation sustains Smo activity thereby causing Gli1 accumulation and nuclear translocation. Pathways leading to increased Sonic transcription remain to be elucidated. Moreover, in samples like the BCPAP cell line, knock-down of Ptc, the receptor that suppresses the pathway, may contribute to pathway activation. Again, wether this occurs at a transcriptional level or is caused by gene deletion remains to be clarified.

Several reports have indicated a cooperative role of Shh pathway with other oncogenic mediators, such as RAS-MAPK and AKT (10, 17). In RAS-induced pancreatic cancer, autocrine (cancer cells) and paracrine (stromal cells) signaling mechanisms have been described (10). In thyroid cancer, both RAS-MAPK and AKT pathway play a major role and it will be interesting to explore their interaction with the Shh one.

Whatever the exact mechanism, our findings suggest that pharmacological blockade of the Shh pathway exerts growth suppressing and cytotocxic activities in thyroid cancer cells more potently than in non tumoral thyroid cells. Importantly, these effects were not oncogene-dependent, being observed in cancer cells harboring RET/PTC1 (TPC1), BRAF V600E (BCPAP, BHT101, 8505C) or neither BRAF or RET/PTC1 (HTH74) (16).

Importantly, three of these cancer cell lines (BCPAP, BHT101, 8505C) had also documented p53 mutation, showing that cyclopamine effects do not require functional p53 and may be exerted also in aggressive thyroid cancers that lack p53 function (1). As a specific inhibitor of Smo, cyclopamine has been shown to slow down the growth of a variety of cancers, by affecting at the same time cancer cells and cancer stem cells. Moreover, the lack of obvious secondary effects of cyclopamine treated adult mice (4), suggests that such treatment may spare normal quiescent stem cells in their niches, likely allowing regeneration of normal tissues after cessation of treatment. Other small molecules can interfere with Shh pathway at multiple levels. Besides cyclopamine, other Shh signaling antagonists that bind to Smo include SANT1 and Cur-61414. Instead, GANT68 and GANT61 are small molecules that inhibit Shh signaling downstream of Smo (11). We propose that inhibition of the Shh pathway might be a potential therapeutic strategy in thyroid cancer, particularly those refractory to conventional therapy.

Finally, Shh has been demonstrated to be important in controlling stemness in diefferent tissues and tumors (e.g. glioma, pancreas, colorectal, endometrial) (4, 8, 12, 19). Many recent reports, suggest that Shh signaling regulates the expression of stemness genes and self renewal of cancer stem cells. Nanog has been identified as a Shh mediator of stemness. Our preliminary data indicate that Nanog is upregulated in thyroid cancer and particulalry in ATC. A recent study has reported the identification of tumor-initiating cells with stem properties in thyroid cancer and particularly in ATC, (18). It will be interesting to investigate whether Shh through Nanog is involved in maintenance of this population of thyroid cancer stem cells.

MATERIALS AND METHODS

Tissue samples

Archival thyroid samples were retrieved from the files of the Department of Oncology of University of Pisa (Pisa, Italy). Informed consent was obtained from the patients, and the study was approved by the institutional review board committee. Tumor size, extrathyroid invasion, node metastasis, associated thyroid lesions, and metastatic deposits were recorded. After surgical resection, tissues were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Sections (4 microns thick) were stained with hematoxylin and eosin for histological examination.

Immunohistochemistry

Formalin-fixed and paraffin-embedded 4- to 5-microns-thick tumor sections were deparaffinized, placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min, and treated with blocking serum for 20 min. The slides were incubated overnight with anti-Gli1 monoclonal antibody (Vectostain ABC kits, Vector Laboratories, Inc., Burlingame, CA). As a negative control, tissue slides were incubated with isotype-matched IgG1 control antibodies. The Gli1 immunostaining was mostly localized in the cytoplasm; the percentage of positive cells for Gli1 staining was evaluated.

Cell culture

Human primary cultures of normal thyroid cells (P5) were obtained from F. Curcio and cultured as previously described (5). Human papillary (TPC1, BCPAP, NIM) and anaplastic (BHT101, HTH74, 8505C) thyroid cancer cell lines have been described previously (14). All the cells were SNP genotyped to ensure correct identity.

NTHY (Nthy-ori 3-1) are normal human thyrocytes immortalized by the Large T of SV40 and were obtained from the European Tissue Culture collection. NTHY and tumor cell lines were grown in the DMEM medium supplemented with either 2.5% or 10% FBS, L-glutamine and penicillin/streptomycin (all reagents were from Sigma, Munich, Germany). Cyclopamine was purchased from Calbiochem and it was used for 48h at 10 and 20 μM concentration for luciferase and biological assays.

DNA constructs

pGL3-Gli-Luc was obtained by PCR-cloning eight copies of the Gli binding element into the pGL3 enhancer vector purchased from Promega. pCEFL Gli1 were obtained by cloning the full length PCR products of Gli1 into the PCEFL vector with the Gateway technology (Invitrogen).

Cell viability measurement

Seventy-two hours after transfection, Cell Titer Blue reagent (CellTiter® Blue Assay, Promega) diluted 1:1 in cell culture medium was added to each well. Plates were incubated at 37 °C for 6 hours with 5% CO_2 and then transfered to room temperature for overnight incubation, at dark. Cell viability was measured with an EnVision Multilabel plate reader (Perkin Elmer). Transfection efficency was previously tested by using the GAPDH Alert kit (Ambion) according to the manifacturer's protocol.

BrdU assay

BrdU (5-bromo-2-deoxyuridine) incorporation assay was performed by using the Cell Proliferation ELISA-BrdU chemiluminescent Kit (Roche Applied Science). Transfected cells were labeled with BrdU, then fixed and incubated with anti-BrdU-Peroxidase solution. Finally, each sample was incubated with a buffered solution containing luminol and BrdU incorporation was quantified by measuring light emission with a microplate luminometer with photomultiplier technology.

Apoptosis assay

The Apo-ONE® Homogeneous Caspase-3 Assay (Promega) was performed in thyroid cancer cell lines. Blank, positive and negative controls were performed as well. Apo-ONE® Homogeneous Caspase-3 Reagent was added 1:1 to cell culture volume. The fluorescence values were measured with an EnVision Multilabel plate reader (Perkin Elmer), proportionally to the amount of caspase-3/7 cleavage activity in each sample.

Immunofluorescence microscopy

For indirect immunofluorescence, TPC1 cells were serum starved for 16–20 hours and treated with cyclopamine (10 μM) for 24 hours. After three washings with PBS, coverslips were placed in a humidified chamber for one hour at room temperature with a 1:100 dilution of Gli-1 and subsequently a FITC-conjugated donkey rabbit secondary antibody (Jackson Immunoresearch). Coverslips were then inverted and mounted onto glass slides with Vectashield containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and viewed with fluorescence microscopy. Images were taken using a SPOT digital camera attached to a Zeiss Axiophot microscope with an 100X objective (Carl Zeiss, Thornwood, NY).

Reporter assays

Luciferase activities present in cellular lysates were assayed using the Dual-Luciferase Reporter System (Promega). In all cases, the total amount of plasmid DNA was adjusted with pCDNAIII-β-gal (a plasmid expressing β-galactosidase). Light emission was quantitated using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Data were represented as luciferase activity present in each sample, and the values plotted were the average ± SEM of triplicate samples from typical experiments, which were repeated at least 3–5 times with nearly identical results.

Real time PCR

The expression of Shh, Ptch, Smo and Gli1 was studied by real-time PCR. Briefly, total RNA was extracted from tissues and cell lines, using the Rneasy Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. 1 μg of total RNA was used as starting material, to which 1 μl oligo-dT was added, as well as DEPC-treated water to total volume of 25 μl. The RNA mix were heated at 65°C for 5 min and then chilled on ice. The other components were added to each RNA mix as follows: 10 μl of 5X first-strand buffer, 5 μl of 0.1 M DTT, 1 μl of 25 mM dNTPs, 1 μl of ribonuclease inhibitor and 6 μl of DEPC-treated water. The samples were incubated at 42°C for 2 min, followed by the addition of 1 μl of Superscript II (40 U/l, Invitrogen) and incubated at 42°C for 50 min. The reaction was inactivated at 70°C for 5 min, followed by the addition of 1 μl (2 U/l) of Rnase H and incubation at 37°C for 20 min. Real-time PCR was performed using the Biorad-iCycler IQ detector system and Biorad-iCycler IQ SYBR Green mix (Biorad), primarily following the manufacturer's protocol. In brief, the reaction mixture (25 μl total volume) contained 500 ng of cDNA, gene-specific forward and reverse primers for each gene at 1 M final concentration and 12.5 μl of 2X SYBR Green mix. The real-time cycler conditions were as follows: PCR initial activation step at 95°C for 10 min, 40 cycles each of melting at 95°C for 15 sec and annealing/extension at 60°C for 1 min. A negative control without template was included in parallel to assess the overall specificity of the reaction. The experiments were repeated in triplicates as suggested by the manufacturer's.

Statistical analysis

The raw data of the siRNA screen were normalized and analyzed with the R/Bioconductor software (7).

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FIGURE LEGENDS

Figure 1: Immunohistochemical detection of Gli1 in thyroid samples.

Representative tissue samples from normal thyroid, PTC and ATC were incubated with a mouse monoclonal anti-Gli1 antibody. ATC samples showed intense immunoreactivity for Gli1, while PTC tissues were stained less intensely and normal tissues were completely negative. Negative controls were performed in all cases using isotype-matching control antibodies.

Figure 2: Expression of Shh components in thyroid tumors.

Q-RT-PCR was used to calculate Shh, Ptc and Gli1 mRNA expression in six independent PTC and ATC tumor samples with respect to a pool of four normal thyroids. The results are the average of three independent experiments.

Figure 3: Up-regulation of Shh pathway in cultured thyroid cancer cells.

A, Semiquantitative RT-PCR (25 cycles) was performed to evaluate mRNA levels of Shh, Ptc, Smo and Gli1 in the indicated cell lines. Actin mRNA detection was used for normalization. This figure is representative of three independent experiments. **B,** Activation of Gli1 transcription factor was tested by Luciferase assay. Thyroid cancer cells, but not normal NTHY cells, activated Gli-Luc reporter when compared to empty pGL3 vector. This signal was abolished by treatment with cyclopamine. Gli1 transfection was used as positive control of reporter activation. **C,** Gli1 is localized in the nucleus of TPC1 cells and cyclopamine is able to reduce both, the total amount and the nuclear fraction of Gli1, as shown by immunofluorescence. Three independent determinations were performed in triplicate. SD are reported.

Figure 4: Cyclopamine treatment effects on thyroid cancer cells.

TPC1, BCPAP, 8505C, HTH74 and NTHY cells were treated with cyclopamine at 10 and 20μM. Measurement of viability by cell titre blu (**A**), proliferation by BrdU incorporation (**B**), and apoptosis by caspase 3 cleavage (**C**) was performed.

Table 1

Hystology	% Gli1 positive cells
Normal thyroid	0
Adenomas	38
FTCs	28
PTCs	60
follicular variant	45
classical variant	80
tall cell variant	90
ATCs	65

Thyroid samples of different hystotypes (10 samples for each group) were stained for Gli1 expression. The percentage of Gli1-positive cells was estimated. The number of positive cells correlated with stain intensity. Average positive cells in the various tissue samples catagories was calculated. Samples were considered positive when containing at least 10% positive cells.

Figure 1

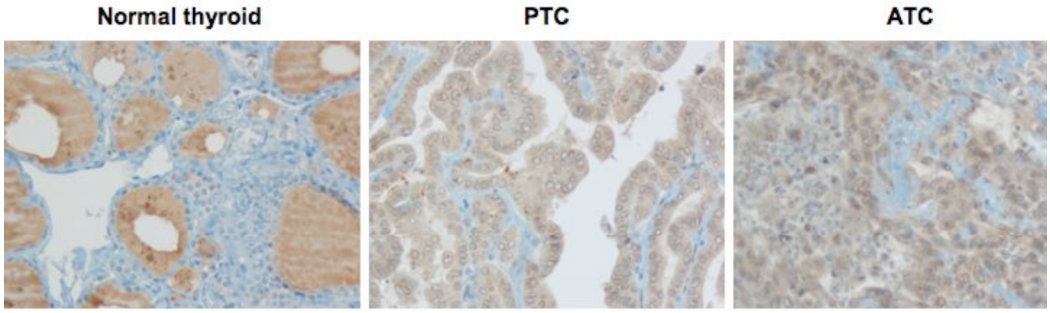


Figure 2

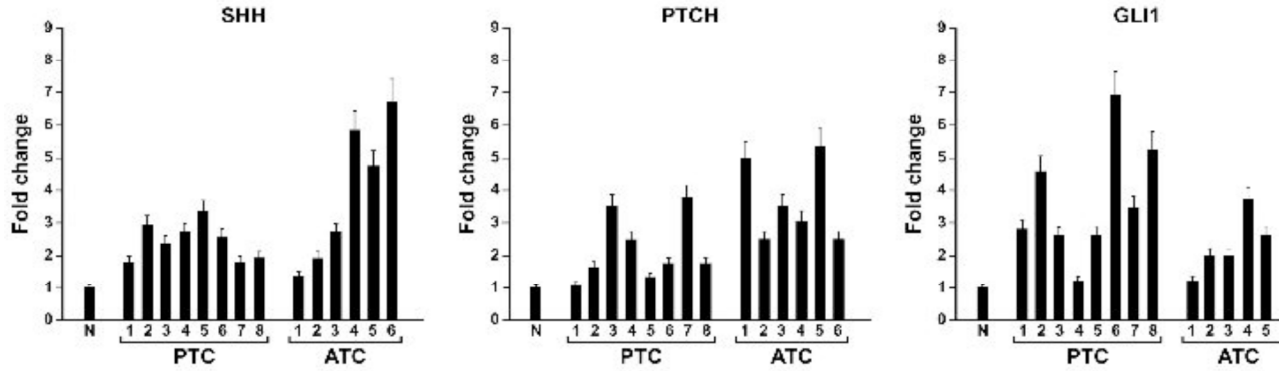
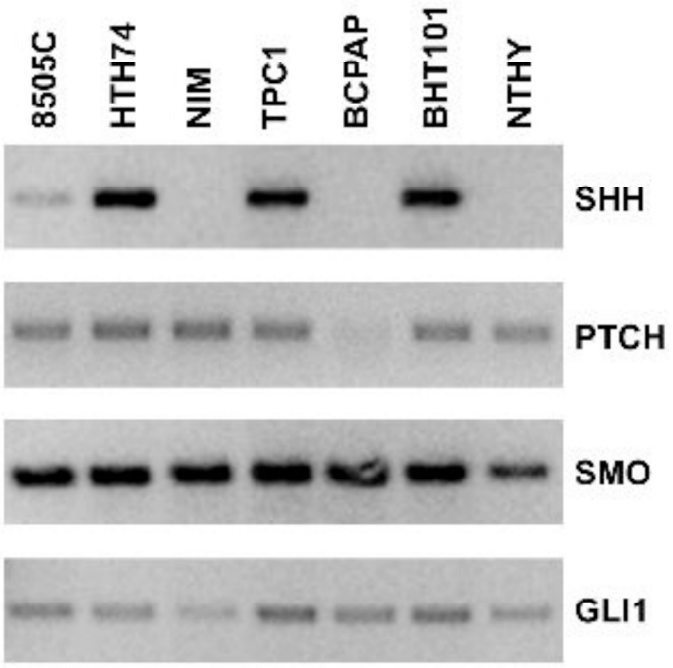
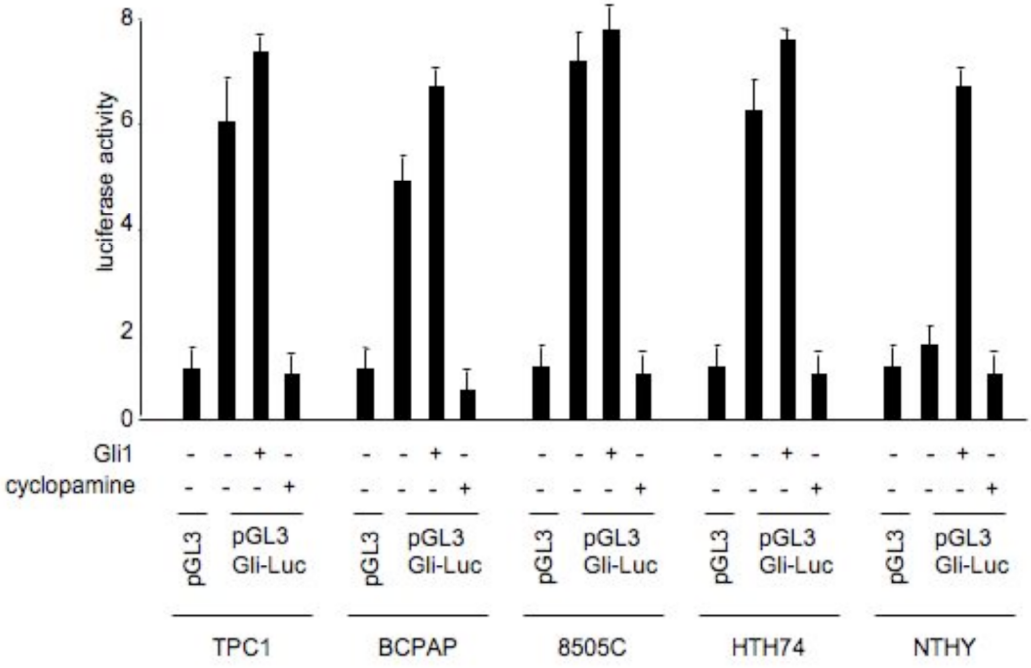


Figure 3

Panel A)



Panel B)


luciferase activity
8
6
4
2
0
Gli1
cyclopamine
pGL3
pGL3 Gli-Luc
TPC1
BCPAP
8505C
HTH74
NTHY

Panel C)

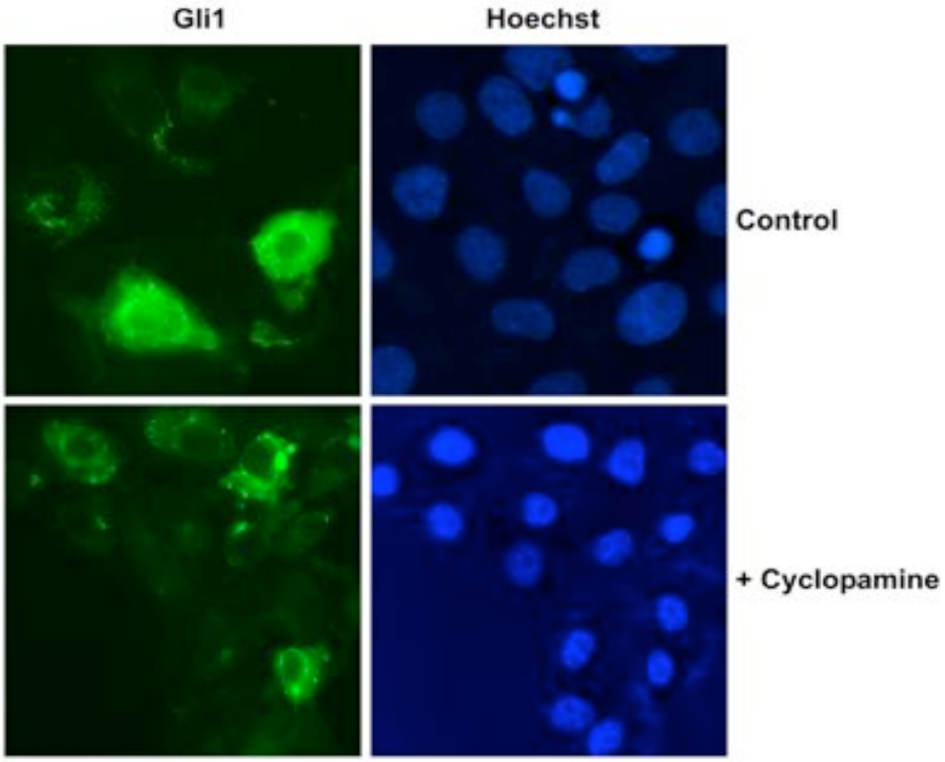
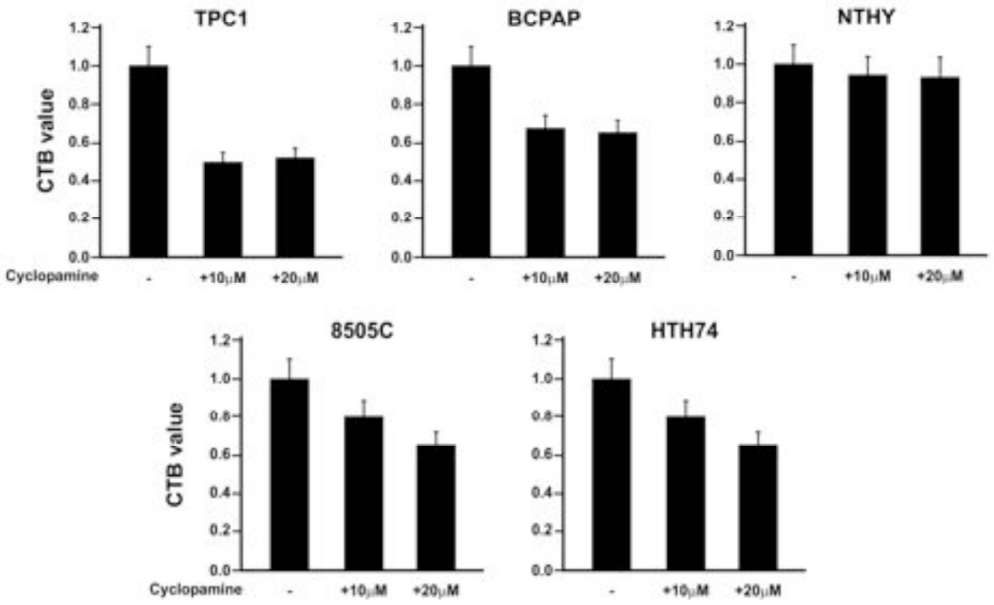
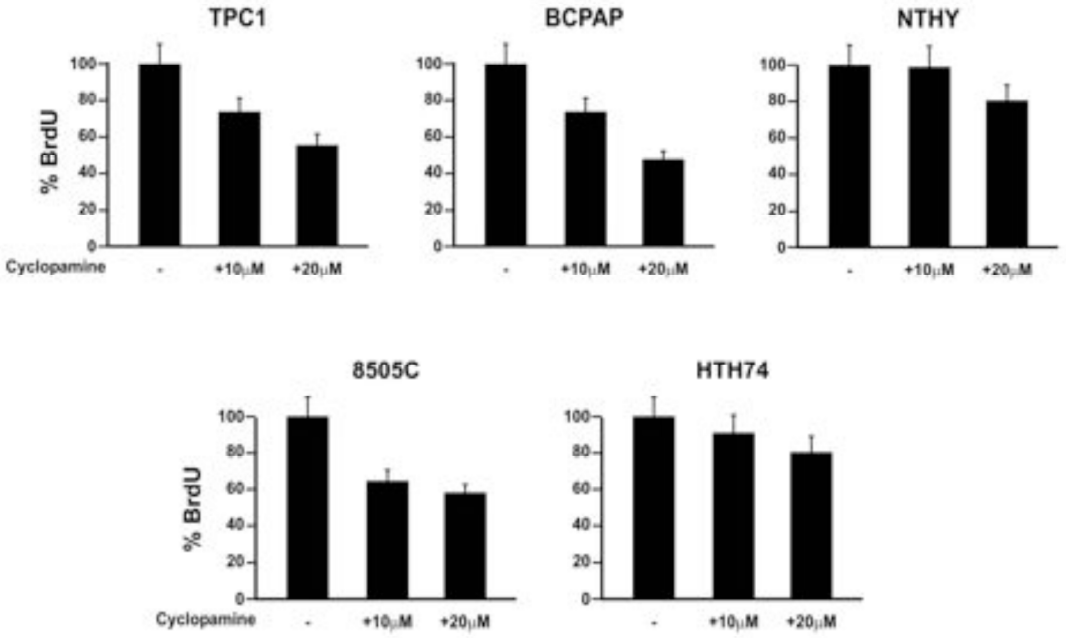


Figure 4

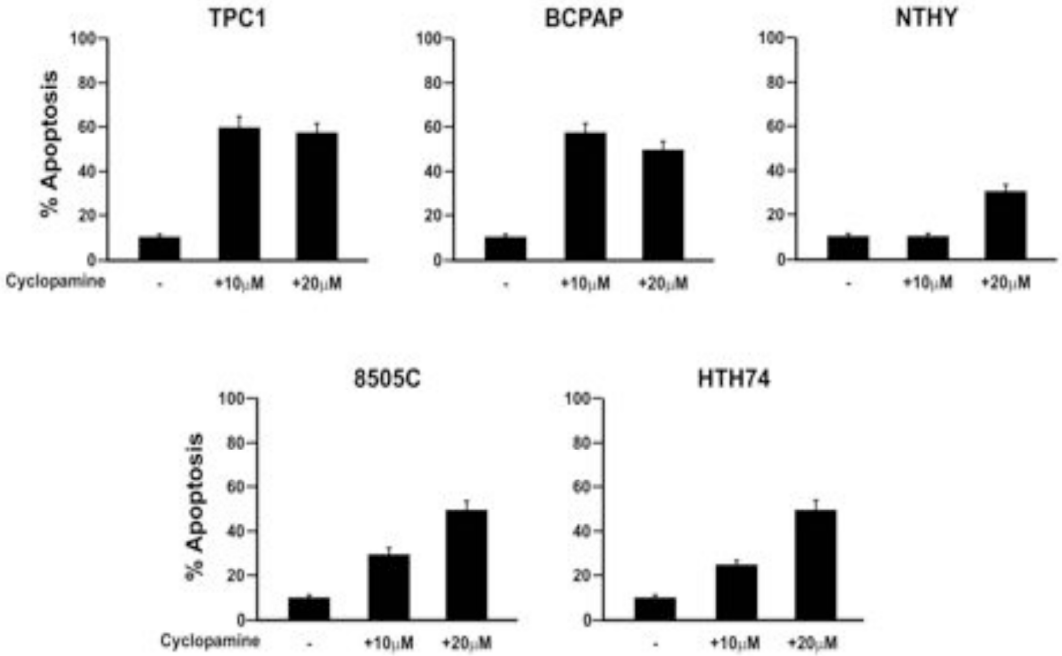
Panel A)



Panel B)


TPC1
BCPAP
NTHY
8505C
HTH74
% BrdU
100
80
60
40
20
0
Cyclopamine
-
+10μM
+20μM

Panel C)


TPC1
BCPAP
NTHY
8505C
HTH74
% Apoptosis
0
20
40
60
80
100
Cyclopamine
-
+10μM
+20μM

Attached manuscript # III

Laatikainen LE, Castellone MD, Hebrant A, Hoste C, **Cantisani MC**, Laurila JP, Salvatore G, Salerno P, Basolo F, Nasman J, Dumont JE, Santoro M, Laukkanen MO.

Extracellular Superoxide Dismutase is a thyroid differentiation marker down-regulated in cancer.

Endocr Relat Cancer. *2010 Aug 16;17(3):785-96*.

Endocrine-Related Cancer (2010) **17** 785–796

Extracellular superoxide dismutase is a thyroid differentiation marker down-regulated in cancer

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Abstract

Reactive oxygen species, specifically hydrogen peroxide (H_2O_2), have a significant role in hormone production in thyroid tissue. Although recent studies have demonstrated that dual oxidases are responsible for the H_2O_2 synthesis needed in thyroid hormone production, our data suggest a pivotal role for superoxide dismutase 3 (SOD3) as a major H_2O_2-producing enzyme. According to our results, *Sod3* is highly expressed in normal thyroid, and becomes even more abundant in rat goiter models. We showed TSH-stimulated expression of *Sod3* via phospholipase C–Ca^{2+} and cAMP–protein kinase A, a pathway that might be disrupted in thyroid cancer. In line with this finding, we demonstrated an oncogene-dependent decrease in *Sod3* mRNA expression synthesis in thyroid cancer cell models that corresponded to a similar decrease in clinical patient samples, suggesting that SOD3 could be used as a differentiation marker in thyroid cancer. Finally, the functional analysis in thyroid models indicated a moderate role for SOD3 in regulating normal thyroid cell proliferation being in line with our previous observations.

Endocrine-Related Cancer (2010) **17** 785–796

Introduction

The G protein-coupled receptor (GPCR) activating $G\alpha_s$ and $G\alpha_q$ proteins, such as TSH receptor (TSH-R), mediate the cellular responses to various extracellular stimuli via two main signal transduction pathways: the cAMP pathway and the phosphatidylinositol pathway that is involved in calcium signaling (Pierce *et al.* 2002). In thyroid, the TSH-R cAMP pathway is responsible for the thyroid cell proliferation, differentiation, and secretion, whereas the TSH-R phosphatidylinositol cascade controls thyroid hormone tri-iodothyronine (T_3) and thyroxine (T_4) synthesis. The TSH is crucially important for normal thyroid function, and therefore, deregulation caused by, e.g. TSH-R mutations or external factors, affecting TSH signaling interferes with the cellular homeostasis in thyroid (Ludgate & Vassart 1995). A low iodide uptake leads to reduced thyroid hormone T_3 and T_4 production (hypothyroidism), which is compensated by increased TSH synthesis and subsequent activation of downstream signaling leading to cell proliferation and benign thyroid enlargement known as goiter (Dumont *et al.* 1989). Similarly, activating mutations of the TSH-R cause autonomous hyperfunctioning thyroid adenomas

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and nonautoimmune toxic thyroid hyperplasia (Duprez *et al.* 1994).

TSH signaling has been shown to increase the synthesis of hydrogen peroxide (H_2O_2), which is used as an extracellular substrate by thyroperoxidase in thyroglobulin iodination and thyroid hormone synthesis (Corvilain *et al.* 1991). On the other hand, intracellular H_2O_2 contributes to mitogen signaling and consequent cellular proliferation in *in vitro* and *in vivo* models (Rao & Berk 1992, Guyton *et al.* 1996, Kuroki *et al.* 1996) by regulating the activation of protein tyrosine phosphatases and tyrosine kinase receptors (Rao 1997, Wang *et al.* 2000, Saito *et al.* 2002, Aslan & Ozben 2003, Konishi *et al.* 2004, Mehdi *et al.* 2005). We have previously demonstrated the ability of extracellular superoxide dismutase (SOD3), which is one of the main H_2O_2-producing enzymes (Marklund 1984, Karlsson & Marklund 1987), to promote cell proliferation *in vivo* by activating Ras–Erk mitogen signaling, transcription factor upregulation and growth factor expression suggesting growth stimulatory role in tissues (Laurila *et al.* 2009).

In the current study, we investigated the signal transduction pathways and synthesis of *Sod3* in different experimental models to determine the function of SOD3-derived H_2O_2 in thyroid. We identified a novel TSH-R-mediated signaling pathway activating *Sod3* synthesis and decreased *SOD3* expression in thyroid malignancies.

Materials and methods

Animals

Male 4–5 weeks old Sprague–Dawley rats (University of Turku, Turku, Finland) were given 0.25% propylthiouracil (PTU; Sigma) *ad libitum* in drinking water for 2 weeks, killed, and tissues were collected for expression analysis. As controls for histological staining, we used nontreated rat thyroids, and for quantitative reverse transcription (qRT)-PCR analysis, a thyroid total RNA pool from four animals. Experimental procedures were done according to the European Union and the University of Turku guidelines (permission 2009/2502).

Histological analysis

Thyroid tissues were embedded in optimal cutting temperature compound, OCT (Tissue-Tek, Torrance, CA, USA). Ten-micrometer sections were stained with hematoxylin/eosin (Sigma) according to the standard protocols.

Cell lines and reagents

Rat PC Cl3 cells were grown in Ham's F-12 medium, Coon's modified (Sigma) supplemented with 5% calf serum (Life Technologies, Inc.) and 10 nM TSH, 10 nM hydrocortisone, 100 nM insulin, 5 μg/ml transferrin, 5 nM somatostatin, and 20 μg/ml glycyl-histidyl-lysine. PC RET/PTC1 and PC E1A stable cell lines, kindly provided by Prof. Alfredo Fusco, were grown in F-12 medium supplemented with 5% calf serum without hormones (Fusco *et al.* 1987, Santoro *et al.* 1993). PC-inducible RET/PTC1 cells were grown in the same medium as PC Cl3 cell administered with 1 μg/ml doxycyclin (Sigma). COS-7 cells were grown in DMEM (Sigma) supplemented with 10% fetal bovine serum (Life Technologies, Inc). Transfections (1 μg of plasmid) were done with Fugene6 (Roche Applied Science) for PC Cl3 cells, and with Polyfect (Sigma) for COS-7 cells. Human *SOD3* cDNA, kindly provided by Prof. Stefan L Marklund from the University of Umeå, Sweden, was cloned to pcDNA3 vector (Life Technologies). The plasmids pCEF-$G\alpha_s$ and pCEF-$G\alpha_q$, encoding constitutive active form (QL) of $G\alpha_s$ and $G\alpha_q$ and pCEF-protein kinase A (*PKA*), encoding the active catalytic subunit of PKA, were kindly provided by Dr Silvio Gutkind from NIH, MD, USA. PC Cl3 cells were incubated with 10 nM TSH (Sigma), 40 μM forskolin (Sigma), 1 μM *N*(2-((ρ-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide (H89; Calbiochem, San Diego, CA, USA), or 100 nM thapsigargin (Research Biochemicals International, Natick, MA, USA). SOD1 protein was purchased from Sigma.

PCRs

Total RNA was isolated from tissues pooled from four animals or cells using Tri-reagent (Sigma). The first-strand synthesis was done with QuantiTect RT (Qiagen) and qPCR with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). Primers were rat *Sod1*-for GTC GTC TCC TTG CTT TTT GC and rat *Sod1*-rev TGC TCG CCT TCA GTT AAT CC; rat *Sod2*-for AAG GAG CAA GGT CGC TTA CA and rat *Sod2*-rev TGG CTA ACA TTC TCC CAG TTG; rat *Sod3*-for GAC CTG GAG ATC TGG ATG GA and rat *Sod3*-rev GTG GTT GGA GGT GTT CTG CT; human *SOD3*-for CTT CGC CTC TGC TGA AGT CT and human *SOD3*-rev GGG TGT TTC GGT ACA AAT GG; human *βACTIN*-for TGC GTG ACA TTA AGG AGA AG and human *βACTIN*-rev GCT CGT AGC TCT TCT CCA; rat *βActin*-for TCG TGC GTG ACT TAA GGA G and rat *βActin*-rev GTC AGG CAG CTC GTA GCT CT. Reactions were done in 60 °C annealing temperature.

Real-time PCR tissue samples

The qRT-PCR expression analyses from human tissues were done to normal thyroid ($n=7$), papilloma thyroid cancer (PTC, $n=9$), and anaplastic thyroid cancer (ATC, $n=6$) patient samples. The PTC patient samples had 30% genetic variation (six wild-type Braf, one Braf V600E, and two RET/PTC1 rearrangements). Five ATC patient samples had Braf V600E mutation and one had wild-type Braf. The analysis of mRNA expression was done from pooled samples.

DNA array tissue samples

The diagnoses of the hyperthyroidic patients were based on the long-standing hyperthyroidism, absence of thyroid-stimulating antibodies in the serum and in some cases, family history and confirmed by DNA sequencing of the TSH-R. The details of the patient material were described earlier (Hebrant *et al.* 2009). Shortly, thyroid samples were obtained from five familial nonautoimmune hyperthyroidism (FNAH) French patients (from Nancy (FNAH1 and FNAH4), Reims (FNAH2), Lyon (FNAH3), and Angers (FNAH5)), autonomous adenoma (AA) samples were obtained and pooled from four different patients as published previously (Hebrant *et al.* 2009).

In order to compare FNAH and AA, we used the reference pool of 23 normal thyroid tissues adjacent to different pathologies. The human HEEBO (human exonic evidence-based oligonucleotide) 70-mer oligonucleotide microarrays, containing ~48 500 probes (representing exonic sequences, alternatively spliced exons, expressed sequence tags (ESTs), and controls) were used for the array analysis. All hybridizations were performed in duplicates with dye swap. For the PTC and ATC microarray, 20 PTCs and 9 ATCs were hybridized on Affymetrix slides (HGU133 plus 2.0), according to Affymetrix protocol. The ethics committees of the institutions have approved protocols according to Declaration of Helsinki.

Calcium uptake assay

Intracellular Ca^{2+} concentrations were measured in PC Cl3 cells. Briefly, cells were detached from tissue culture plates with PBS containing 0.5 mmol/l EDTA, washed once with HEPES-buffered medium (HBM; 137 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $CaCl_2$, 0.44 mmol/l KH_2PO_4, 4.2 mmol/l $NaHCO_3$, 10 mmol/l glucose, 20 mmol/l HEPES, and 1.2 mmol/l $MgCl_2$, pH 7.4), and then loaded with 2 μmol/l fura-2 acetoxymethyl ester for 20 min at 37 °C. After loading, the cells were diluted with HBM without $CaCl_2$ to a final concentration of 0.3 mmol/l $CaCl_2$ and stored at room temperature until use. For fluorescence recordings, an appropriate volume of the cell suspension was spun down, washed once in HBM, resuspended in HBM, and placed in a thermostated (37 °C) cuvette with magnetic stirring in a Hitachi F-2000 fluorescence spectrophotometer. The fluorescence was monitored at 340 nm (excitation) and 505 nm (emission). Experiments were calibrated with 60 μg digitonin/ml (F_{max}) and 10 mmol/l EGTA (F_{min}). The intracellular $[Ca^{2+}]$ was calculated from the fluorescence (F) using the equation $[Ca^{2+}]=(F-F_{min})/(F_{max}-F)\times 224$ nmol/l (K_d for fura-2), in which the extracellular fura-2 fluorescence was subtracted from F values.

RNA interference

Short interfering *Sod3* or *Gapdh* OnTargetplus SMART pool oligos (Dharmacon, Lafayette, CO, USA) were transfected into PC Cl3 cells according to the manufacturer's protocol. Shortly, 20 μmol/l of siSOD3 or siGAPDH control oligo were resuspended to total volume 100 μl transfection buffer, mixed with equal volume of Optimem (Life Technologies), and incubated 5 min at room temperature. The optimation of small interference RNA (siRNA) quantity used in the study was based on the siGAPDH silencing effect using KDalert GAPDH assay kit (Ambion, Austin, TX, USA). To prepare the transfection solution, 5 μl Dharmafect 4 reagent was added to 190 μl of Optimem and incubated 5 min at room temperature. Oligo suspension and transfection reagent were combined, incubated 20 min at room temperature, and added to the cells for 48 h.

Bromodeoxyuridine analysis

Cells were serum starved for 16 h and TSH stimulated for 6 h before the addition of 10 mM bromodeoxyuridine (BrdU; Roche) for 2 h. Subsequently, cells were fixed in 3% paraformaldehyde (Sigma) and permeabilized with 0.2% Triton X-100 (Sigma). BrdU-positive cells were revealed with FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cell nuclei were identified by Hoechst (Sigma) staining.

Chemiluminescence assay for $O_2^{\cdot-}$ detection

Extracellular $O_2^{\cdot-}$ release was detected by chemiluminescence using the SOD-inhibitable Diogenes reagent (National Diagnostics, Atlanta, GA, USA) on cells resuspended in Hanks' Balanced Salt Solution (HBSS)/10 mmol/l glucose. One μmol/l ionomycin was added to the cells to stimulate the dual oxidase (DUOX)

activity. Measurements were taken with 22 s intervals for 10 min in 96-well white plates (1.5×10^5 cells/250-μl well) at 37 °C using a Microplate Luminometer. Chemiluminescence curves were analyzed, and peak values of the curves were presented in Relative Light Units/s.

Fluorimetric assay for H_2O_2 detection

The H_2O_2 release was quantified 48 h after cell transfection by the homovanillic acid-based fluorimetric assay (Benard & Brault 1971) and by 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen). For homovanillic assay, the cells were incubated in Krebs–Ringer–HEPES medium pH 7.4 containing 0.1 μg/ml HRP type II, 440 μmol/l homovanillic acid, and 1 μmol/l ionomycin during 2 h 30 min at 37 °C. Fluorescence intensity of oxidized homovanillic acid was measured at 425 nm after excitation at 315 nm. Results are reported as ng of H_2O_2/well representing the quantity of H_2O_2 accumulated during 2 h 30 min in each well (six-well plates). For the H_2DCFDA assay, the cells were grown in 96-well dishes, the reagent (1 mg/ml) was added to the cells for 15 min, and the fluorescence was measured with A_{380}/A_{435}.

Statistical analysis

Statistical analysis was done using *t*-test for means ± S.D.

Results

SOD3 is abundantly expressed in rat thyroid tissue and stimulated by TSH

SOD3 is an extracellular H_2O_2-producing enzyme expressed in various tissues (Marklund 1984, Laukkanen *et al.* 2000) protecting cell surface structures against deleterious effects of superoxide. The local administration of *Sod3*, causing twofold increased total SOD activity in tissues, or i.v. infusion of the native C-form of the isolated recombinant enzyme with affinity to cell surface structures results in significant attenuation of tissue damages and markedly increased tissue recovery (Hatori *et al.* 1992, Sjoquist & Marklund 1992, Laukkanen *et al.* 2002), suggesting that even minor changes in tissue SOD3 concentration have a significant impact on the redox balance.

Comparison of SOD isoenzyme mRNA expressions from normal rat tissues (Fig. 1A) demonstrated thyroid as one of the major expression sites for *Sod3*. Accordingly, mRNA extraction from normal rat tissues showed 2.5-fold higher expression levels in thyroid as compared with heart, corresponding to a previously reported threefold and fivefold differences in thyroid versus heart protein level in rabbit and human respectively (Marklund 1984). The expression in the rat thyroid approached the levels detected in the aorta, which is one of the main expression sites for SOD3 (Laukkanen *et al.* 2002), indicating participation to ROS balance and signal transduction in thyroid tissue. The expression of *Sod1* and *Sod2* was in line with previously reported enzyme activities in the rat tissues (Marklund 1984).

Because SOD3 activation increases H_2O_2 synthesis that has been shown to be up-regulated in response to TSH, we used a rat *in vivo* model to investigate the expression status of SOD3 in benign thyroid enlargement caused by increased TSH secretion and to compare the animal data to human clinical samples. PTU treatment is known to cause decreased T_3 production with subsequent increase in TSH, thyroid enlargement, and activated thyroid function (Levey 1963, al-Alawi *et al.* 1995, Laezza *et al.* 2006); we therefore treated four rats with PTU *ad libitum* and killed the animals after 2 weeks to isolate the thyroids. As shown in Fig. 1, PTU-treated thyroids had on average fourfold increase in wet-weight (animals 4–7) as compared with normal untreated animals (rat 1–3; Fig. 1B), suggesting a typical goiter formation, which was further supported by hematoxylin–eosin staining demonstrating increased cellular division and loss of follicular lumen (Fig. 1C and D). The variation seen in thyroid growth is in line with previous reports (Levey 1963) due to *ad libitum* administration of PTU. To study the expression of *Sod3* mRNA, we pooled in two separate groups the normal and the treated rats, and showed tenfold increased ($P<0.01$) mRNA synthesis in PTU-treated animals (Fig. 1E), thereby suggesting that the enzyme production is activated by TSH signaling. However, the DNA array analysis of human AA and FNAH samples failed to demonstrate significant differences between patient groups (Fig. 1F), suggesting that the human phenotype is milder than the phenotype of PTU-treated rats.

We then extended our analysis to clinical thyroid patient samples by comparing the SOD isoenzyme expression in normal human thyroid (Fig. 1G) and found that in humans, *SOD1* showed highest degree of expression differently from the rat thyroid samples (Fig. 1A). Finally, in order to confirm the significance of SOD3 in thyroid cell physiology, we compared the expression of *Sod1*, *Sod2*, and *Sod3* in a normal thyroid rat cell line (PC Cl3). According to qRT-PCR data, *Sod3* expression was significantly higher as compared with *Sod1* ($P<0.01$) and *Sod2* ($P<0.01$; Fig. 1H) validating the *in vivo* observations in the rat model (Fig. 1A).

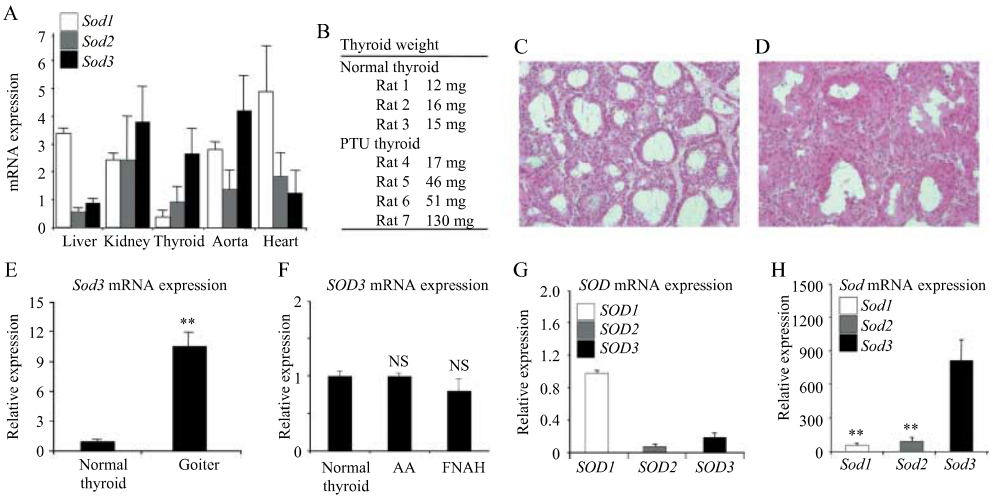


Figure 1 Superoxide dismutase expression in rat and human tissues. (A) Quantitative RT-PCR analysis for *Sod1*, *Sod2*, and *Sod3* mRNA expression from normal untreated rat tissues. The analysis suggested high expression level for *Sod1* in liver, kidney, aorta, and in heart; for *Sod2* in kidney; and for *Sod3* in kidney, thyroid, and aorta. (B) Thyroid weight measurement from three normal and four PTU-treated rats. Rats 1–3: normal controls, rats 4–7: PTU-treated animals. The analysis showed approximately fourfold increase in wet weight of the PTU-treated tissues indicating goitrogenous development. The use of PTU *ad libitum* for 14 days in drinking water caused variation seen in thyroid weight development. (C and D) Hematoxylin–eosin staining for control rats (C) and for PTU-treated rats (D) showed hyperplasia and altered thyroid structure caused by the treatment. (E) *Sod3* mRNA expression upon PTU treatment. The *Sod3* levels in rat thyroid were significantly ($P<0.01$) increased by PTU treatment. (F) Human DNA array analysis from normal thyroid, autonomous adenoma (AA), and from familial nonautoimmune hyperthyroidism (FNAH) showed insignificant differences for *SOD3* mRNA expression. (G) Real-time RT-PCR analysis for *SOD* mRNA expression from normal human thyroid samples. In human thyroid tissue, *SOD1* represents the major isoenzyme differently from rat thyroid tissue shown in panel 1A. (H) Comparison of *Sod1*, *Sod2*, and *Sod3* expression in rat thyroid PC Cl3 cells by real-time RT-PCR analysis suggested *Sod3* as a major isoenzyme in the cell line being in line with normal rat thyroid expression analysis shown in panel 1A. *Sod3* synthesis was significantly ($P<0.01$) higher than *Sod1* and *Sod2* mRNA production.

TSH signal transduction increases *Sod3* mRNA expression

The upregulation of SOD3 in rat hyperfunctioning thyroids suggests that SOD3 could be under the control of TSH, and therefore, we investigated the effect of the different mediators of TSH signaling on regulating *Sod3* production in rat PC Cl3 cell line. As shown in Fig. 2A, the SOD3 mRNA synthesis was significantly induced by TSH stimulation (threefold increase, $P<0.01$). Because TSH-R is coupled to both G proteins: to $G\alpha_s$, which causes accumulation of cAMP and activates PKA, and to $G\alpha_q$, which increases the intracellular Ca^{2+} level and activates protein kinase C (PKC), we studied the contribution of $G\alpha_s$-mediated pathway on *Sod3* mRNA production by transiently transfecting constitutively active $G\alpha_s$ (pCEF-$G\alpha_s$) and PKA (pCEF-*PKA*) into PC Cl3 cells that resulted in 5.5-fold ($P<0.01$) and sixfold increase ($P<0.001$) in SOD3 mRNA synthesis respectively. Similarly, accumulation of cAMP after the activation of adenylyl cyclase with forskolin promoted *Sod3* mRNA transcription of sixfold ($P<0.05$), supporting the involvement of TSH–cAMP pathway in the production of SOD3. To further demonstrate the specificity of *Sod3* activation, we inhibited the PKA function in TSH-stimulated PC Cl3 cells by using a selective PKA inhibitor, H89. Interestingly, even though TSH–H89 incubation significantly decreased ($P<0.05$) *Sod3* production, it was not able to completely abolish the mRNA induction, suggesting the contribution of other signaling routes in SOD3 regulation (Fig. 2A). Because in thyroid cells TSH-derived signaling is also mediated via $G\alpha_q$–PLC–Ca^{2+} signal transduction pathway (Song *et al.* 2007), we transfected PC Cl3 cells with an active form of $G\alpha_q$ (pCEF-$G\alpha_q$) or incubated the cells with thapsigargin, which increases calcium influx. Transfection of pCEF-$G\alpha_q$ as well as treatment with thapsigargin increased relative mRNA production by threefold ($P<0.01$ and $P<0.05$ respectively; Fig. 2B and C), suggesting that both $G\alpha_s$/cAMP/PKA and $G\alpha_q$/PKCγ/Ca^{2+} signal transduction pathways are involved in SOD3 regulation in PC Cl3 cells. The effect of thapsigargin on promoting the Ca^{2+} levels in PC Cl3 cells was determined by measuring the intracellular Ca^{2+} concentrations at different time points, upon treatment with the compound (Fig. 2C).

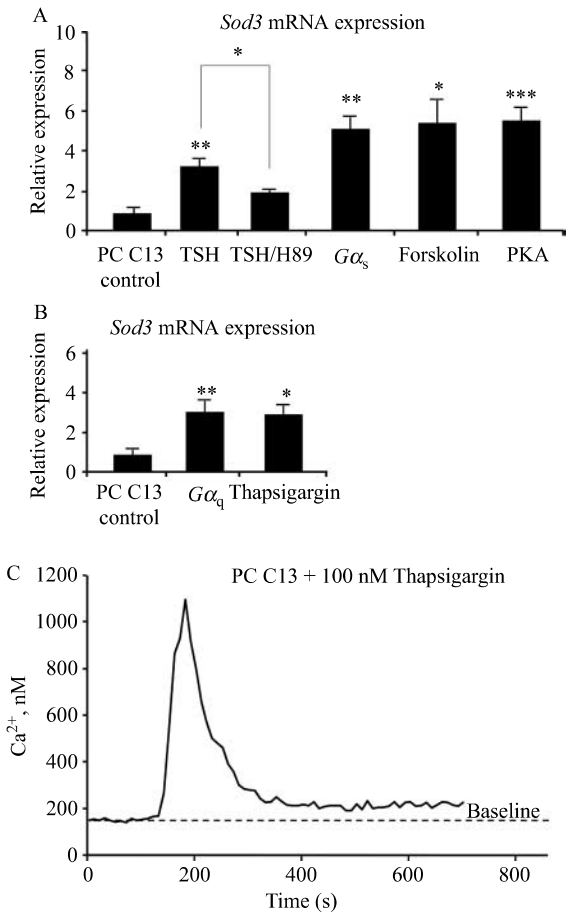


Figure 2 TSH activation increased *Sod3* mRNA production in PC Cl3 cells. (A) TSH-R cAMP pathway increased *Sod3* production. The expression was analyzed by qRT-PCR in cells treated with TSH and forskolin or transfected with *PKA* or $G\alpha_s$, which all induced the mRNA synthesis significantly ($P<0.01$, $P<0.05$, $P<0.001$, and $P<0.01$ respectively). Treatment with PKA inhibitor H89 reduced significantly ($P<0.05$) the effect of TSH but was not able to completely inhibit the expression. (B) The effect of TSH-R phophatidylinositol cascade on *Sod3* synthesis was studied by $G\alpha_q$ subunit transient transfection and by calcium uptake analysis using thapsigargin that blocks the calcium pumps in endoplasmic reticulum increasing the cytosolic calcium concentration. Both treatments, transfection and thapsigargin administration, increased the mRNA synthesis significantly ($P<0.01$ and $P<0.05$ respectively). (C) The intracellular calcium levels upon thapsigargin treatment were measured by fluorescent calcium uptake assay. The levels went promptly up after 100 nmol/l thapsigargin administration and stayed above the baseline values over the follow-up period.

SOD3 has a mitogenic effect in thyroid cells

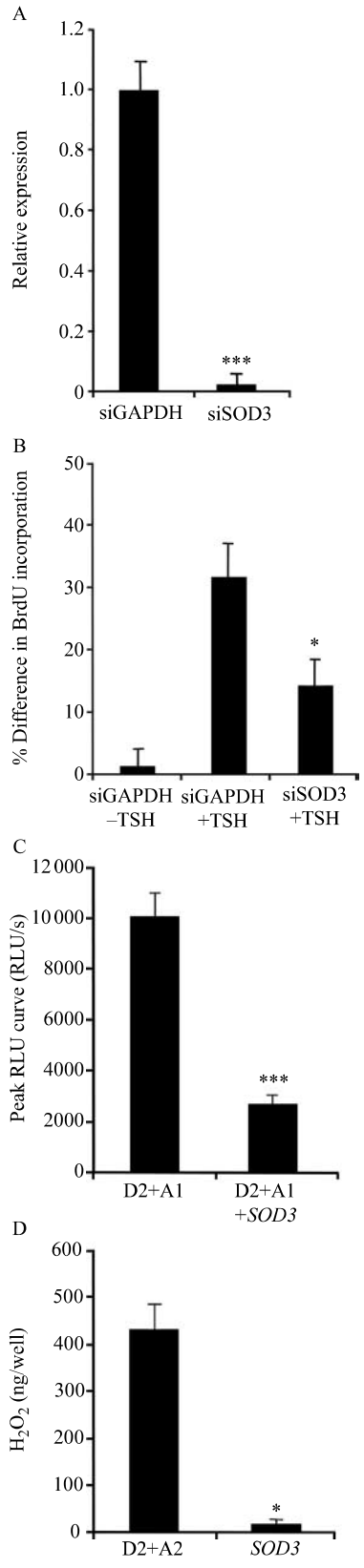
We then aimed to investigate the physiological consequences of TSH-stimulated *Sod3* production in thyroid cells. In addition to antioxidative characteristics, we have recently shown that exogenously administered *Sod3* is able to stimulate Ras GTP loading *in vitro* and *in vivo* leading to the activation of mitogen signaling pathway and consequent growth factor expression and cell proliferation (Laurila *et al.* 2009). This is further supported by numerous reports showing that physiological concentrations of H_2O_2 can activate cell membrane receptors and downstream signaling leading to cell proliferation *in vitro* and *in vivo* (Rao 1997, Wang *et al.* 2000, Saito *et al.* 2002, Aslan & Ozben 2003, Konishi *et al.* 2004, Mehdi *et al.* 2005).

Since *Sod3* is highly expressed in PC Cl3 cells (Fig. 1H), the overexpression of *SOD3* did not have any detectable effect on cell proliferation (data not shown). We therefore investigated the effect of RNAi on normal rat thyroid cell proliferation. The efficiency of *Sod3* siRNA transfection was checked by qRT-PCR analysis, showing 95% interference effect ($P<0.001$) in *Sod3* mRNA production at 48-h time point (Fig. 3A). Importantly, by transfecting siRNA for *Sod3* (siSOD3) into TSH-stimulated PC Cl3 cells, we formally proved that SOD3 has a role in regulating the mitogenic effect of TSH on thyroid cells, as the transfection of siSOD3 significantly ($P<0.05$) reduced the *in vitro* BrdU incorporation from 33 to 15% (Fig. 3B). This mitogenic effect of SOD3 is in line with our earlier observations (Laurila *et al.* 2009).

Because previous studies have shown that the production of H_2O_2 in thyroid cells correlates with the stimulation of T_3 and T_4 production (Corvilain *et al.* 1994), we determined the dismutase activity of SOD3 in Cos-7 heterologous system to predict the participation of SOD3 in hormone synthesis. For this, we used control cells expressing DUOX2 (D2) and the activator of DUOX1 (DUOXA1 or A1), which together produce $O_2^{\cdot-}$ rather than H_2O_2 (Zamproni *et al.* 2008, Morand *et al.* 2009). Transiently transfected *SOD3* showed significant ($P<0.001$) reduction in extracellular $O_2^{\cdot-}$ concentration indicating functionality of SOD3 in our cellular model (Fig. 3C). Finally, because DUOXes have been indicated as the main sources for H_2O_2 production in thyroid, we compared H_2O_2 levels produced by DUOX and SOD3 (Fig. 3D), and found that indeed, the SOD3-derived H_2O_2 production was 28-fold lower than by DUOX2–DUOXA2 pair ($P<0.05$), suggesting a role for SOD3-mediated H_2O_2 production in controlling TSH-dependent thyroid growth and differentiation rather than in hormone production.

SOD3 is down-regulated in thyroid cancer

In line with the observation that SOD3 could be involved in the TSH-dependent proliferation/dedifferentiation process, we decided to investigate the expression of *Sod3* in malignant thyroid proliferative disorders, firstly by determining the mRNA



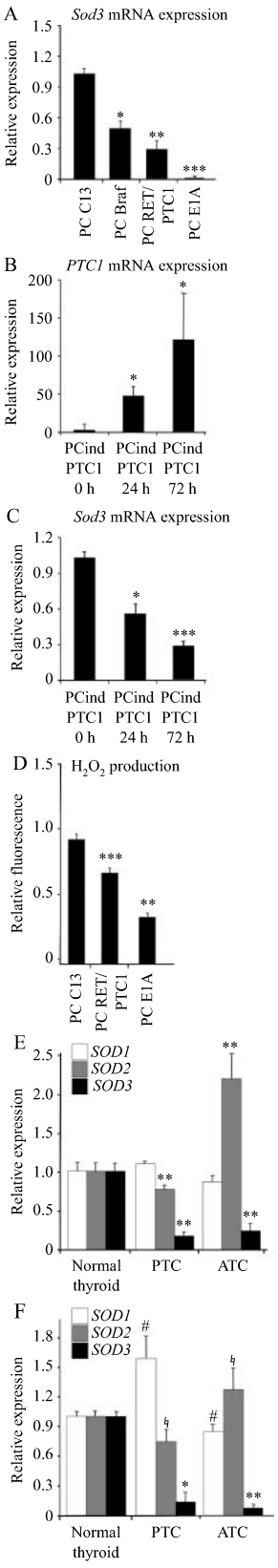
levels from three rat PC Cl3 derived models of oncogene-dependent dedifferentiation, PC Braf, PC RET/PTC1, and PC E1A stable cell lines, which showed decreased *Sod3* synthesis in correlation with oncogene-mediated transformation and dedifferentiation as compared with normal PC Cl3 cells (Fig. 4A). PC Braf cells showed twofold decreased ($P<0.05$) mRNA synthesis, PC RET/PTC1 cells showed threefold decreased ($P<0.01$) *Sod3* mRNA amount where as PC E1A cell model lacked almost completely the expression ($P<0.001$) as compared with control PC Cl3 cells.

To further study the effect of oncogene induced inhibition of SOD3 expression, we took advantage of an inducible oncogenic cellular model, doxycyclin *RET/PTC1*-inducible PC cells. As shown in Fig. 4B and C, the increased expression of the *RET/PTC1* oncogene upon doxycyclin treatment correlated with a significant reduction in *Sod3* expression at 24- and 72-h time points ($P<0.05$ at 24 h and $P<0.001$ at 72 h), indicating that the different levels of *Sod3* mRNA downregulation observed in Fig. 4A could also be due to the different expression levels of the specific oncogenes.

Since H_2O_2 represents the main product of *Sod3* activation, we confirmed the decreased *Sod3* expression in PC RET/PTC1 and PC E1A cells by measuring the H_2O_2 levels, which were significantly ($P<0.001$ and $P<0.01$ respectively) decreased in transformed cells as compared with wild-type cells (Fig. 4D).

To finally move our observation to human models, we then compared DNA array data obtained from normal human thyroid, PTC, and ATC tissues, and showed significantly ($P<0.01$) decreased *SOD3* synthesis in papillary thyroid tumors and ATC tissue ($P<0.01$) in line with *in vitro* cell models (Fig. 4A). These data were further confirmed by qRT-PCR analysis, which gave similar results (Fig. 4F).

Figure 3 Functional role of SOD3 in thyroid models. (A) RNAi knockdown of *Sod3* in PC Cl3 cells. Quantitative RT-PCR showed significant ($P<0.001$) *Sod3* downregulation caused by siSOD3 interfering oligos. (B) BrdU incorporation analysis suggested significantly ($P<0.05$) attenuated cell proliferation caused by siSOD3 in TSH-stimulated PC Cl3 cells. The TSH-stimulated proliferation was decreased by 44% in siSOD3-transfected cells as compared with siGAPDH-transfected cells. (C) The functionality of *SOD3* to dismutase DUOX2/DUOXA1 (D2+A1) derived superoxide was tested in Cos-7 heterologous system. The data showed that SOD3 was able to dismutase the DUOX2/DUOXA1 produced superoxide significantly ($P<0.001$) indicating functionality of the enzyme in this cell model. (D) SOD3-derived hydrogen peroxide production was tested in Cos-7 heterologous system. Properly folded DUOXA2 produced significantly ($P<0.05$) higher concentration of H_2O_2 than SOD3. Transfection of *SOD3* resulted in only minor increase in H_2O_2 synthesis suggesting that DUOX enzyme complex has the major role H_2O_2 synthesis in thyroid.



The expression of other isoenzymes, *SOD1* and *SOD2*, were more variable in patient samples; *SOD1* expression was mildly affected by tumorigenesis, whereas *SOD2* was decreased in PTC patients ($P<0.01/P<0.05$, DNA array/qRT-PCR) and significantly increased in ATC thyroid tumors ($P<0.01/P<0.05$, DNA array/qRT-PCR). The *in vitro* and *in vivo* data therefore showed that SOD3 is expressed in normal thyroid tissue, induced by TSH together with thyroid differentiation markers (Dumont *et al.* 1992, Kondo *et al.* 2006), and reduced in *in vitro* transformation models and thyroid cancer samples.

Discussion

Thyroid tissue physiology requires continuous high concentration of H_2O_2 for thyroid hormone T_3 and T_4 synthesis which is, according to recent publications, produced mainly by DUOX (Dupuy *et al.* 1991, 2000, De Deken *et al.* 2002, Rigutto *et al.* 2007). Inactivating mutations of DUOX2 completely abolish H_2O_2 generation and thyroid hormone synthesis in human thyroids, and constitute a major cause of congenital hypothyroidism (Moreno *et al.* 2002). SODs are H_2O_2-producing isoenzymes that are responsible for balancing the reduction–oxidation reactions in different cellular compartments. In thyroid tissue, H_2O_2 is utilized in thyroid hormone production by transmembrane glycoprotein thyroperoxidase that both

Figure 4 *SOD3* expression decreases upon thyroid cell transformation. (A) Quantitative RT-PCR analysis for *Sod3* mRNA from wild-type rat PC Cl3 cells and PC Cl3 cells transformed with different oncogenes indicated decreased mRNA production correlating to the oncogene used for cell transformation. The *Sod3* synthesis decreased 50% ($P<0.05$) in PC Braf cells, 60% ($P<0.01$) in PC RET/PTC1 cell model, and was almost completely abrogated ($P<0.001$) in PC E1A cells. (B and C) PC-inducible RET/PTC1 (PCindPTC1) cell model showed that the decreased *Sod3* mRNA synthesis depends on the level of oncogene expression. The doxycyclin-induced *RET/PTC1* oncogene expression (B) correlates to the decreased *Sod3* production (C). (D) Determination of H_2O_2 production. The fluorimetric measurement of H_2O_2 synthesis showed decreased H_2O_2 production in PC RET/PTC1 ($P<0.001$) and PC E1A ($P<0.01$) cells as compared with PC Cl3 controls being in line with the reduced expression of the *Sod3* enzyme. (E) Analysis of DNA array data from normal thyroid samples derived from papillary thyroid (PTC) and anaplastic thyroid (ATC) cancers showed reduced *SOD3* expression in transformed tissues ($P<0.01$). The differences of *SOD1* expression were statistically insignificant, whereas the expression of *SOD2* was significantly ($P<0.05$) decreased in PTC samples and significantly ($P<0.01$) increased in ATC patients. (F) Real-time RT-PCR analysis supported the array data, showing that only *SOD3* but not the other isoenzymes could be considered as a differentiation marker in cancer development. Both analyses, DNA array and qRT-PCR, showed that the expression of *SOD1* and especially *SOD2* was more variable.

oxidizes and incorporates iodine to thyroglobulin, which is then further metabolized to thyroid hormones T_3 and T_4. In the present study, we therefore investigated the expression and role of SODs, more specifically extracellularly expressed SOD3, in thyroid cells *in vitro* models and further compared the results to patient samples data.

The expression analysis of different rat tissues suggested thyroid as a major expression site for the enzyme (Fig. 1). According to our previous studies, relatively low concentration of SOD3 *in vitro* or *in vivo* is able to induce a physiological response in the cells and in the surrounding extracellular tissue environment (Laukkanen *et al.* 2000, 2001*b*, 2002, Laurila *et al.* 2009), suggesting that even minor differences in the enzyme concentration can have an impact on cellular signaling and consequent biological effects.

We studied the *SOD3* expression in benign thyroid enlargement models to determine the expression of the enzyme in the proliferative environment. Even though previous clinical analysis has demonstrated decreased total SOD activity in the plasma of Graves' disease patients (Wilson *et al.* 1989, Abalovich *et al.* 2003, Rybus-Kalinowska *et al.* 2008), a recent paper suggested increased *Sod3* mRNA production in thyroid caused by long-term iodine deprivation in murine models (Maier *et al.* 2007). Since both the synthesis of autoantibodies against TSH-R in Graves' disease and the diet iodine deprivation lead to increased TSH-R signaling and consequent thyroid activation, we investigated the *Sod3* expression in experimental *in vivo* rat goiter model (Fig. 1). Increased *Sod3* production in rat goiter model was in line with previous murine models (Maier *et al.* 2007), suggesting the participation of TSH in its expression in mouse and rat thyroid cells. However, analysis of patient samples did not show significant increase in *SOD3* levels, which could be due to the existence of wide phenotypic variations in humans, ranging from mild to severe toxic hyperfunction of the gland. It is also noteworthy that unlike in rat normal thyroid and in rat PC Cl3 thyroid cells, in normal human thyroid tissue, *SOD1* expression was fivefold higher than *SOD3* mRNA production again indicating species-related differences (Fig. 1).

Because the TSH signaling is the main mediator of thyroid hyperfunction, we studied the effects of the different molecules of this pathway on *Sod3* mRNA synthesis. TSH signals to TSH-R, a GPCR that activates $G\alpha_s$ and $G\alpha_q$ heterotrimeric G-proteins in human and rodents (Laugwitz *et al.* 1996). Activation of $G\alpha_s$ leads to the stimulation of cAMP, a positive modulator of thyroid cell proliferation and the expression of differentiation markers such as sodium iodide symporter and thyroperoxidase (Kogai *et al.* 1997, Pierce *et al.* 2002, van Staveren *et al.* 2006). The $G\alpha_q$ protein stimulation in thyroid activates DUOX-derived H_2O_2 production, which is the limiting factor in thyroglobulin iodination and in thyroid hormone T_3 and T_4 synthesis (Corvilain *et al.* 1994). To determine the role of *Sod3* in thyroid *in vitro* model, we utilized the PC Cl3 cell line that according to our present data contains higher concentration of SOD3 than other isoenzymes and is therefore responding to SOD3-mediated redox changes (Fig. 1H). By studying the effect of TSH signaling in these cells, we identified a novel GPCR-mediated induction of *Sod3* through both, a cAMP and phosphatidylinositol-mediated way, suggesting a role for the enzyme in thyroid physiology (Fig. 2). Since GPCR-mediated signal transduction plays a significant role also in other tissues, the current data could potentially further explain previous observations of SOD3-mediated effects in injury models (Laukkanen *et al.* 2002, Brasen *et al.* 2007).

In order to investigate the functional effect of the enzyme in thyroid environment, we used siRNA and overexpression of *SOD3* in heterologous Cos-7 model to study the proliferation and level of H_2O_2 production respectively (Fig. 3). According to the present data, knockdown of *Sod3* is able to attenuate TSH-stimulated proliferation proving a role for the

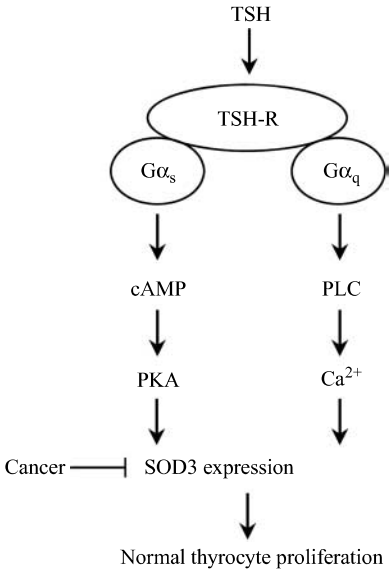


Figure 5 Scheme for activation of *Sod3* transcription in thyroid cells. TSH stimulation leads to signal transduction via cAMP–PKA and PLC–Ca^{2+} routes that both increase *Sod3* mRNA synthesis. In normal thyroid, the increased *Sod3* production has a role in thyrocyte proliferation where as in thyroid cancer the signal transduction route leading to increased *Sod3* levels is silenced, as a result of the loss of differentiation markers.

enzyme in normal thyroid proliferation. In heterologous Cos-7 model, the transfected *SOD3* reduced extracellular superoxide generation by mismatched *DUOX1* and *DUOXA2*, but the SOD3 derived H_2O_2 was significantly lower than the H_2O_2 produced by matched *DUOX2* and *DUOXA2* at equal levels of expression, suggesting that, differently from DUOXes, SOD3 is not directly regulating thyroid hormone synthesis.

Finally, because the TSH signaling, as a differentiation marker, is down-regulated in cancer, we analyzed different PC Cl3 derived cancer cellular models and showed reduced expression of the *Sod3* mRNA, depending on both the specific oncogene used to transform the cells and the level of the oncogene expression (Fig. 4A–C).

To further analyze the SOD expression in thyroid tumorigenesis, we determined the mRNA expressions from human patient samples. Similarly to *in vitro* models, in both, papillary and anaplastic cancer tissue samples, we observed a reduced *SOD3* expression, suggesting that this gene behaves as a thyroid differentiation marker reduced in malignant transformation (Fig. 4E and F).

In conclusions, we have shown a novel TSH stimulation-dependent GPCR-mediated signal transduction pathway activating *Sod3* mRNA production in rat thyroid PC Cl3 cell line. We have further demonstrated differentiation-dependent *SOD3* expression both *in vitro* and *in vivo* in patient samples. The finding suggests that SOD3 is involved in TSH mitogen effect in normal thyrocytes, a pathway that is disrupted in thyroid cancer (Fig. 5).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L E Laatikainen, M D Castellone, A Hebrant, C Hoste, M C Cantisani, J P Laurila, G Salvatore, P Salerno, F Basolo, J Näsman, and M O Laukkanen contributed to the experimental part of the work; M D Castellone, J Näsman, J E Dumont, M Santoro, and M O Laukkanen contributed to the design of the experiments and editing of the manuscript; J E Dumont, M Santoro, and M O Laukkanen provided funding for the research.

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