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”Role of Sonic Hedgehog signaling in thyroid tumorigenesis and tumor-stroma interaction”

Alessia Parascandolo

University of Naples Federico II
Dipartimento di Medicina Molecolare e Biotecnologie Mediche

Administrative Location

Dipartimento di Medicina Molecolare e Biotecnologie Mediche
Università degli Studi di Napoli Federico II

Partner Institutions

- Università degli Studi di Napoli "Federico II", Naples, Italy
- Istituto di Endocrinologia ed Oncologia Sperimentale "G. Salvatore", CNR, Naples, Italy
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“Role of Sonic Hedgehog signaling in thyroid tumorigenesis and tumor-stroma interaction”

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2. MC Cantisani, A Parascandalo, M Perälä, C Allocca, V Fey, N Sahlberg, F Merolla, F Basolo, MO Laukkanen, OP Kallioniemi, M Santoro, MD Castellone – A Loss-of-Function Genetic Screening Identifies Novel Mediators of Thyroid Cancer Cell Viability, Oncotarget, ***in press.***

3. G Vecchio, A Parascandolo, C Allocca, A Strazzulli, M Moracci, C Ugolini, F Basolo, MD Castellone, M Santoro, N Tsuchida - Downregulation of FUCA-1 in human thyroid anaplastic carcinomas - ***in preparation***

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette transporter
AKT	v-Akt murine Thymoma viral oncogene
ALDH	Aldehyde Dehydrogenase
ATC	Anaplastic Thyroid Cancer
BCC	Basal Cell Carcinoma
BCL2	B-cell lymphoma 2
BRAF	B-type RAF family kinase
CAF	Cancer Associated Fibroblast
CCDC6	Coiled Coil Domain Containing 6
CK1ε	Casein Kinase 1ε
CNV	Copy Number Variation
CSC	Cancer Stem Cell
CycD	D-type cyclin
Dhh	Desert Hedgehog
ECM	Extracellular Matrix
EMT	Epithelial-Mesenchymal Transition
FAPα	Fibroblast Activation Protein α
FDA	Food and Drug Administration
FTC	Follicular Thyroid Cancer
FV-PTC	Follicular Variant-PTC
GANT	Gli Antagonist
GBM	Glioblastoma Multiforme
Gli	Glioma-Associated Oncogene
GSK3β	Glycogen Synthase Kinase 3 β
HDACis	Histone Deacetylase inhibitors
HPI	Hedgehog Pathway Inhibitor
IC50	Half maximal Inhibitory Concentration
IGF	Insulin-like Growth Factor
Ihh	Indian Hedgehog
MAPK	Mitogen-Activated Protein Kinases
MEN2	Multiple Endocrine Neoplasia type 2
MF	Myelofibrosis
MSC	Mesenchymal Stomal Cell
MTC	Medullary Thyroid Cancer
MYC	Myelocytomatosis oncogene
NCOA4	Nuclear Receptor Coactivator 4
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PAX8	Paired Box 8
PDA	Pancreatic Ductal Adenoma
PDGF	Platelet-derived growth factor
PDTC	Poorly Differentiated Thyroid Cancer

PI3K	Phosphoinositide-3 kinase
PKA	Protein Kinase A
PPARγ	Peroxisome-Proliferation Activated Receptor γ
PTC	Papillary Thyroid Cancer
Ptch1	Patched receptor
PTEN	Phosphatase and Tensin Homolog
Q-RT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RET	REarranged during Transfection
RET/PTC	RET/Papillary Thyroid Carcinoma
SCR	Scrambled sequence
Shh	Sonic Hedgehog
SMANT	Smo Mutant Antagonist
Smo	Smoothned receptor
Sufu	Suppressor of Fused
TCV-PTC	Tall Cell Variant-PTC
TERT	Human Telomerase reverse transcriptase
TGF-β	Transforming growth factor-β
TKI	Tyrosine Kinase Inhibitor
Tn-C	Tenascin-C
TP53	Tumor Protein 53
TRH	Thyrotropin-Releasing-Hormone
TSH	Thyroid-Stimulating-Hormone
VEGF	Vascular Endothelial Growth Factor
WDTC	Well-Differentiated Thyroid Cancer
α-SMA	α-Smooth Muscle Actin

ABSTRACT

Thyroid carcinomas represent the most common endocrine cancers with their incidence increasing worldwide. Up to date there are no efficient treatments for patients affected by metastatic thyroid disease that is not responsive to radioiodine treatment. Sonic Hedgehog (Shh) pathway is active in regulating embryonic development of different organs as well as of the thyroid gland. The aberrant activation of Shh signaling has been found in several types of cancer and, according to recent evidences, it represents an important regulator of tumor-stroma interaction. In this dissertation, we have studied expression, activation and molecular mechanisms regulating the Shh pathway in thyroid cancer and its involvement in the modulation of tumor stroma interaction in anaplastic thyroid cancer cells (ATC). Our results suggest that Shh signaling is activated through different mechanisms in thyroid cancer cells: in a non-canonical way in cooperation with other oncogenic pathways to promote and support thyroid neoplastic transformation as well as in a paracrine way in tumor-stroma interaction, supporting thyroid cancer cells invasion and migration. Our data therefore suggest that Shh signaling could be a novel therapeutic target in aggressive thyroid cancers.

1.0 BACKGROUND

1.1 Thyroid gland

The thyroid is the largest endocrine gland, located on the anterior-inferior region of the neck. It consists of two conical lobes joined by a transverse segment, the isthmus. The gland is composed of functional units, the follicles, which are delimitated by an epithelial cuboidal cell layer and filled with colloid (the major constituent of the thyroid mass, representing a thyroglobulin protein reserve) (Williams textbook of Endocrinology 2003). The follicular cells, also called thyrocytes, are involved in iodine uptake and production of thyroid hormones. The parafollicular cells (C cells) represent the second thyroid cell population, blended among the follicular cells, producing the calcium-regulating hormone calcitonin (Kondo et al. 2006). The thyroid gland has an active role in the regulation of several biological functions through the synthesis and secretion of thyroid hormones, L-tri-iodothyronine (T3), the active hormone, and L-thyroxine (T4) (De Lellis et al. 2004). The hypothalamic-pituitary axis finely regulates thyroid hormones production through Thyrotropin-Releasing-Hormone (TRH) produced by the hypothalamus and acting on the pituitary gland stimulating the synthesis of Thyroid-Stimulating-Hormone (TSH). The TSH hormone stimulates the follicular cells that undergo changes in their morphological features, from cuboidal to columnar, and secrete thyroid hormones (Kondo et al. 2006; Williams textbook of Endocrinology 2003).

1.2 Thyroid carcinoma

Thyroid carcinoma, the most common endocrine cancer, is classified on the basis of its cellular origins, histological appearance and prognosis (De Lellis et al. 2004). Follicular thyroid cell-derived tumors account for the majority of thyroid cancers and include well-differentiated carcinoma (WDTC), which retains many features of thyroid differentiation, poorly differentiated carcinoma (PDTC) and anaplastic carcinoma (ATC) that is completely undifferentiated highly aggressive and therapy resistant (Cooper et al. 2009). Well-differentiated carcinoma is further classified in papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC). PTC represents 80% of all thyroid cancers whereas FTC represents only a small fraction of thyroid cancers (~15%) (The Cancer Genome Atlas Research Network 2014, Schlumberger and Caillou 2000). However, PTC and FTC may de-differentiate and lead to more aggressive thyroid cancers (The Cancer Genome Atlas Research Network 2014, Bhaijee and Nikiforov 2011). Parafollicular C cell-derived medullary thyroid cancer (MTC) accounts for only 2-5% of thyroid cancers (Wells et al. 2015).

1.2.1 Follicular cell derived thyroid carcinoma

Papillary thyroid carcinoma (PTC) is characterized by papillary structures, which replace the normal thyroid follicular structures, and by typical nuclear features, such as a hypodense chromatine (ground-glass nuclei) (Vu-Phan and Koenig 2014). Several histological PTC subtypes exist, such as the follicular variant (FV), characterized by a follicular growth pattern (The Cancer Genome Atlas Research Network 2014) and the tall cell variant (TCV) consisting of cells that have a height that is three times than their width (Al-Brahim and Asa 2006; Lloyd et al. 2011; LiVolsi 2010). The mitogen-activated protein kinase (MAPK) signaling pathway has an important role in the regulation of thyroid cell proliferation, survival and tumorigenesis. Different genetic alterations of genes encoding mediators of the MAPK signaling pathway have been identified in PTC. BRAF is a serine-threonine kinase member of the RAF family proteins, involved in the MAPK signaling cascade (Nikiforov and Nikiforova 2011; Vu-Phan and Koenig 2014). The BRAFV600E mutation is the most frequent genetic alteration in the classic papillary variant of PTC (~45%) and it is also found in tall cell variant PTC, while it is rare in the follicular variant PTC (Xing 2005; Adeniran et al. 2006). The BRAFV600E point mutation involves a thymine to adenine substitution in the nucleotide position 1799, which leads to a valine to glutamate substitution in the 600 amino acid residue (Ciampi and Nikiforov 2005; Xing 2005). This mutation causes the BRAF constitutive activation and hence the MAPK pathway prolonged stimulation (Cantwell-Dorris et al. 2011; Wan et al. 2004). The AKAP9/BRAF rearrangement, present only in a small fraction of PTCs (1-2%), is due to a paracentric inversion of chromosome 7q leading to the fusion between the BRAF protein kinase domain and the A-kinase anchor protein 9 (AKAP9) gene (Ciampi et al. 2005). RET is a cell surface receptor tyrosine kinase, normally expressed in the developing central and peripheral nervous system (Schuchardt et al. 1994), and its gene is located on chromosome 10q11.2. In thyroid, RET is normally expressed in para-follicular but not in follicular thyroid cells (Bhaijee and Nikiforov 2011; Kondo et al. 2006). Alterations of this gene have been found in ~15% of PTC (Vu-Phan and Koenig 2014) and are characterized by chromosomal rearrangements where the 3'- terminal sequence of RET, encoding the tyrosine kinase domain, is fused *in frame* to 5'- terminal sequence of heterologous genes, forming the chimeric RET/PTC oncogenes. Several RET/PTC variants have been described in PTC, differing for the RET-gene fusion partners. RET/PTC1 and RET/PTC3 are the most frequent RET rearrangements in PTC (Santoro et al. 1994); in RET/PTC1, RET tyrosine kinase domain is fused with the coiled-coil domain-containing gene 6 (CCDC6, also known as H4), in RET/PTC3, instead, the tyrosine kinase domain of RET is fused with the nuclear receptor co-activator 4 (NCOA4, also known as ELE1) (Ciampi and Nikiforov 2005; Santoro et al. 2006). The RET partners share some common features: a) they are expressed in thyroid follicular cells and provide an

active promoter for the expression of rearranged RET tyrosine kinase domain; b) they contain protein-protein dimerization motifs and mediate ligand-independent dimerization and activation of the truncated RET protein (Tong et al. 1997), leading to the activation of several intracellular signalings, such as the MAPK and PI3K-AKT pathways through signaling adaptors recruitment to the phosphorylated Tyr1062 which is localized on the intracellular domain of the RET/PTC protein (Miyagi et al. 2004; Hayashi et al. 2000). The intracellular G-protein RAS regulates important cellular processes such as growth, differentiation and survival. RAS is a small GTP-ase involved in the signal transduction from cell membrane receptor tyrosine kinases and G-protein coupled receptors through the MAPK and PI3K-AKT pathway activation (Nikiforov and Nikiforova 2011). Three RAS genes encode four homologous G-proteins, HRAS, located on chromosome 11p11, KRAS4A ad KRAS4B (resulting from an alternative splicing), located on chromosome 12p12 and NRAS located on chromosome 1p13 (Downward J 2003). Gain-of-function point mutations of HRAS and NRAS have been found in about 15% of PTCs, in particular in the follicular variant PTC (Adeniran et al. 2006; Zhu et al. 2003).

Follicular thyroid cancer (FTC) is the second most common well-differentiated thyroid carcinoma. FTC is characterized by thyroid follicular organization in the absence of papillary structures and typical nuclear features. The similar histological features make the differential diagnosis between follicular adenoma and FTC sometimes difficult (Grebe and Hay 1995; Vu-Phan and Koenig 2014). Nonetheless, genetically PTCs and FTCs are quite different. An important oncogenic alteration found in FTC is the PAX8-PPARγ chromosomal rearrangement. Paired box 8 (PAX8) gene, located on chromosome 2, encodes a transcription factor involved in thyroid development driving the expression of many thyroid-specific genes. The peroxisome proliferator-activated receptor γ (PPARγ) gene is located on chromosome 3 and encodes a member of steroidal hormones-binding nuclear receptor transcription factor family able to form heterodimers with the retinoid X receptor (RXR). It is an important regulator of adipogenesis and usually, it is expressed at low levels in thyroid (Vu-Phan and Koenig 2014). The PAX8-PPARγ rearrangement results from t(2;3)(q13;p25) chromosomal translocation which leads to the fusion of the promoter and most of the PAX8 gene to the coding exons of the PPARγ gene (Kroll et al. 2000). The resulting fusion protein is composed by the PAX8 DNA binding domain and PPARγ receptor and has been shown to be a dominant negative inhibitor of wildtype PPARγ transcriptional activity (Kroll et al. 2000; Yin et al. 2009). PPARγ has often been found down-regulated in different types of thyroid cancers suggesting a tumor suppressive function. Therefore the fusion protein may support the thyroid tumorigenesis also through repressing the PPARγ target genes transcription (Vu-Phan and Koenig 2014). This rearrangement has been found in ~35% of FTC and, at a smaller fraction, in follicular variant PTC as well as in follicular adenoma (Eberhardt et al. 2010).

Finally, constitutively activating RAS mutations, in codon 61 of NRAS and HRAS genes in particular, are present in ~ 40% of FTCs (Nikiforov 2011).

Poorly differentiated thyroid carcinoma (PDTC) is a follicular cell derived carcinoma that represents a morphological intermediate between well-differentiated thyroid carcinoma and undifferentiated (anaplastic) thyroid carcinoma and accounts for 10% of all thyroid cancers (Volante et al. 2010; DeLellis et al. 2004). Activating point mutations of the NRAS gene (codon 61) are the most frequent genetic alteration found in PDTC (Volante et al. 2009). The BRAFV600E point mutation occurs in 5-20% of PDTC and is mainly found in PDTC that derives from PTC rather than PDTC arising from FTC (Nikiforova et al. 2003; Soares et al. 2011). TP53 is a nuclear transcription factor involved in cell cycle and apoptosis regulation. TP53 mutations, in the majority of cases on exons 5-9, occur in 40-50% of PDTC and seem to be an important step to promote progression and dedifferentiation of thyroid tumors (Soares et al. 2011). Mutations of PI3K-AKT pathway effectors have also been found in PDTC, in particular activation of oncogenes such as PIK3CA (5-14%) and AKT1 (5-10%) or inactivation of tumor suppressor genes such as PTEN (20%) (Nikiforov and Nikiforova 2011).

Anaplastic thyroid carcinoma (ATC) is the most aggressive form of thyroid cancer. ATC may result as the last step of the gradual thyroid cancer progression from a well-differentiated to an undifferentiated carcinoma or it can arise de novo. ATC accounts for 1-2% of all thyroid tumors. TP53 loss of function mutations are common genetic events involved in the tumor progression and occur at high rate (50-80%of cases) in ATC (Ricarte-Filho et al. 2009). Activating point mutations of RAS gene occur in 40 to 60% of cases (Pita et al. 2014) while the BRAFV600E point mutation has been found in up to 40% of cases (Ricarte-Filho et al. 2009). Both mutations are important events involved in the dedifferentiation process, although additional genetic alterations are required to promote tumor progression (Nikiforov YE 2004). Mutations of genes involved in the PI3K-AKT pathway, in particular mutations of PI3KCA gene (5-25%), PTEN (5-15%) and AKT1 (5-10%), have been identified in ATC (Rosai et al. 2015; Garcia-Rostan et al. 2005). Amplification of PIK3CA locus in 3q26.3 has been found in 40% of ATC (Ragazzi et al. 2014). Other frequent and important genetic alterations found in ATC are represented by TERT (human telomerase reverse transcriptase) promoter activating mutations. In particular, two mutations located at -124 and -146 bp upstream from the ATG start site (−124 G>A and −146 G>A) have been detected in up to 50% of ATCs (frequently occurring together with BRAFV600E point mutation) (Horn et al. 2013; Rosai et al. 2015). Finally, an important involvement of miRNAs in thyroid tumorigenesis has been shown. Some miRNAs, in particular miR-200 and miR-30 family, have been found down-regulated in ATC, suggesting a

critical role in the acquisition of aggressive tumor features (Fuziwara and Kimura 2014; Visone et al. 2007).

1.2.2 Parafollicular cell-derived thyroid carcinoma

Medullary thyroid cancer (MTC) arises from parafollicular thyroid cells and accounts for 5% of all thyroid cancers (Wells et al. 2013). MTCs occur in hereditary form (25%) as the main component of the hereditary Multiple Endocrine Neoplasia type 2 (MEN2) syndromes, together with pheochromocytoma, while in 75% of cases they occur in sporadic form. Both hereditary and sporadic MTCs are characterized by RET activating point mutations. MEN2 syndrome is classified in MEN2A (85%) and MEN2B (15%) (Wells et al. 2013; Hedayati et al. 2016). In MEN2A RET germline mutations in codons 609, 611, 618, or 620 of exon 10 or codon 634 of exon 11 occur in a high number of cases (Raue and Frank-raue 2012). In MEN2B, RET germline mutations occur in exon 16 (M918T) in ~95% of cases and, only in the 5%, in exon 15 (A883F) (Smith et al. 1997). RET mutations are present in ~50% of sporadic MTC (Marsh et al.1996). Somatic mutations of HRAS, KRAS and rarely NRAS have been found in 18-80% of RET negative sporadic MTCs (Ciampi et al. 2013).

1.3 Role of stroma in thyroid cancer

A growing body of evidences indicates the stroma as a key player in tumorigenesis to support the growth, maintenance and progression of cancer. The constant bidirectional interaction between epithelial cancer cells and stromal components coordinate tumor cell growth, migration, invasion, metastasis and drug resistance (Mueller et al 2004). Cellular microenvironment is composed of several cell types such as cancer associated fibroblasts (CAFs), endothelial cells, immune cells, adipocytes and stem cells interacting in a dynamic manner with extracellular matrix (ECM) in the regulation of tumorigenesis. Stroma composition changes among the different histotypes of thyroid cancers; in PTC it is mainly characterized by immune/inflammatory cells, while in ATC and MTC as well as in metastatic PTC it has been described a desmoplastic stromal reaction, correlating to aggressiveness and lymph node metastasis (Koperek et al. 2007; Koperek et al. 2013). More in detail, the production of collagen type I mediated by stromal fibroblasts seems to be regulated by thyroid cancer cells which induce procollagen type I mRNA translation in neighboring stromal cells in a paracrine fashion (Dahlman et al. 2002). Anaplastic thyroid cancer cells are able to stimulate stromal cells producing growth factors and cytokine such as Platelet-derived growth factor (PDGF), involved in the stromal response (Forsberg et al. 1993), together with induction of transforming growth factor-β (TGF-β) which has been demonstrated to be involved in the desmoplastic reaction in scirrhous gastric cancer (Mahara et al. 1994). Activated stromal

fibroblasts are characterized by three molecular markers namely, Fibroblast activation protein α, (FAPα), Tenascin-C (Tn-C) and α-smooth muscle actin (α-SMA). In the MTC stromal compartment all three fibroblast activation markers are highly expressed compared to stromal compartment of normal thyroid gland with a significant correlation between degree of desmoplasia and expression of activation markers (Koperek et al. 2007). Moreover, up-regulation of FAPα and Tn-C has been found in metastasizing PTCs compared to non-metastasizing PTCs. Desmoplastic stromal reaction seems to be significantly associated to invasive behavior of papillary thyroid microcarcinomas and, in combination with calcification, it is associated to lymph node metastasis (Koperek et al. 2011; Koperek et al. 2013).

1.4 Sonic Hedgehog signaling pathway

The Sonic Hedgehog pathway plays an important role in regulating cell growth, differentiation and tissue patterning during normal human embryonic development. It can act as morphogen in developing tissues in a dose-dependent manner through the induction of different effects on various cell types and it may act as a mitogen that regulates cell proliferation of developing organs (Ingham and McMahon 2001). In adults this pathway is mainly quiescent but has important functions in the regulation of tissue homeostasis, continuous renewal and repair of adult tissues and stem cell maintenance (Hooper and Scott 2005). The Shh signaling pathway has also been recognized to be one of the most important signaling pathways involved in several types of human cancer where it promotes growth, enables proliferation of tumor stem cells and regulates tumor-stroma interaction (Heretsch et al. 2010).

Shh signaling pathway activation is typically initiated in the target cell by binding secreted hedgehog ligands to the protein receptor Patched (Ptch), a 12-span-transmembrane protein (**Fig.1**). In mammals the Hh-genes encode three ligands i) Sonic Hedgehog (Shh), the most extensively characterized, ii) Indian Hedgehog (Ihh) iii) Desert Hedgehog (Dhh), which are characterized by different spatial and temporal distribution patterns (Ingham and McMahon 2001). In vertebrates the Shh pathway localizes at the primary cilium, a slim microtubule-based organelle that protrudes from the cellular surface. In particular, Ptch receptor as well as the seven-span-transmembrane receptor Smoothned (Smo) show dynamic trafficking in cilia depending on Shh ligand binding (Goetz and Anderson 2010; Rohatgi et al. 2007). In the absence of Shh ligand, the Ptch receptor is localized to the base of the cilia and inhibits Smo (located on the membrane of the intracellular endosomes) by preventing its localization to the cell surface (Gupta et al. 2010); upon Shh binding, Ptch looses its inhibitory activity on Smo, which, in turn, moves into the cilium and transduces the Shh signaling downstream. Finally, Ptch receptor is internalized and degraded. On the cell surface there are further regulators that increase or

reduce Shh ligands binding to Ptch receptor. Hhip protein is a negative regulator that competes with Ptch for Shh ligand binding; on the contrary, positive regulators like immunoglobulin (Ig)/fibronectin (Fn)-repeat-containing proteins Boc and Cdo, together with glycosidyl-phosphatidylinositol (GPI)-anchored membrane-bound protein Gas1 favour binding of Shh ligands to Ptch (Heretsch et al. 2010). Shh ligands binding to Ptch receptor triggers a cascade of downstream events that culminates in the activation of the zinc finger transcription factors glioma-associated oncogene Gli. There are three different Gli proteins in mammals, Gli1 that acts as a constitutive activator, Gli2 and Gli3 both containing an activator and repressor domains. Gli proteins are characterized by five highly conserved tandem zinc fingers, conserved N-terminal domain, several PKA phosphorylation sites, and additional small conserved regions in the C-terminal domain. Activated Gli proteins (GliA) translocate into the nucleus where they mediate the transcription of different target genes such as D-type cyclins, B-cell lymphoma 2 (Bcl2), vascular endothelial growth factor (VEGF), myelocytomatosis oncogene (Myc), snail family zinc finger (Snail) and Nanog homeobox (Nanog) (Scales and de Sauvage 2009). Gli transcription factors also mediate Gli1 and Ptch transcription, the last one functioning as a negative feedback in the Shh pathway regulation. In the absence of Shh ligand Gli transcription factors are sequestered in the cytoplasm by a negative regulator protein, Suppressor of fused (SuFu), that targets Gli for phosphorylation mediated by protein kinase A (PKA), glycogen synthase kinase 3 (GSK3β) and casein kinase 1 (CK1ε). Phosphorylated Gli proteins, bound to the adapter protein βTrCP, are subjected to ubiquitination mediated by Cul1-based E3 ligases and are completely degraded (in the case of Gli1 and Gli2) or partially degraded (in the case of Gli3), generating repressive forms (Gli3R) (Wen et al. 2010; Pan et al. 2006).

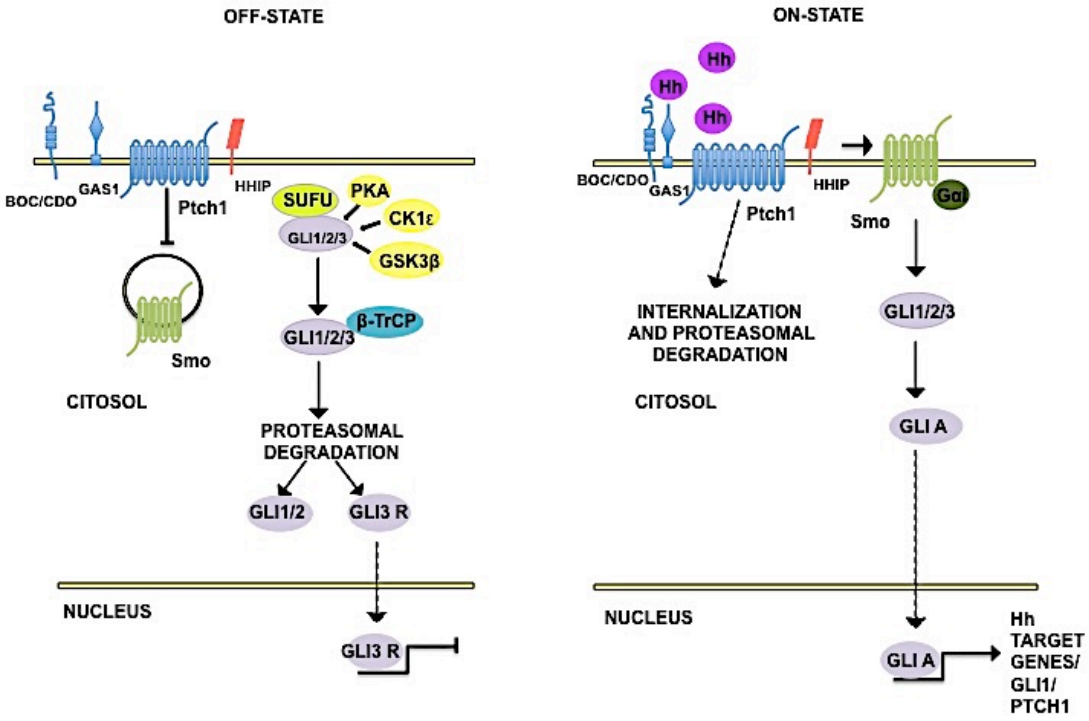


Figure 1. Schematic representation of Hedgehog signaling. Shh pathway cascade is triggered in receiving cell by Shh ligand binding to Ptch1 receptor. In the absence of Shh ligand (Off-State), Ptch1 inhibits Smo receptor localization to the cell surface. Full length Gli proteins are phosphorylated and ubiquitinated to be proteolytically processed and generate the repressor Gli R (mainly derived from Gli3) that prevent Shh target genes transcription. In the presence of Shh ligand binding, Ptch1 receptor is internalized and degraded. Shh ligand binding can be repressed by Hhip and supported by Boc/Cdo and Gas1. Active Smo receptor promotes generation of GliA (mainly derived from Gli1 and Gli2) that is able to translocate to the nucleus where it activates Shh target genes transcription.

1.4.1 Mechanisms of Sonic Hedgehog pathway activation in cancer

Shh pathway alteration has been seen in several types of human cancer and three models of activation have recently been described (**Fig.2**).

Type I Sonic Hedgehog signaling: ligand independent (mutation driven).

Type I ligand-independent model of activation is characterized by loss-of-function mutations in the negative regulators Ptch or SuFu, or gain-of-function mutations in Smo receptor (**Fig.2A**). These events lead to the constitutive activation of Shh pathway in a cell-autonomous manner in the absence of the ligand, and this activation is alone sufficient for tumor initiation and maintenance of different cancers (Scales and Sauvage 2009). For example, in Gorlin's syndrome (or basal cell nevus syndrome) inherited inactivating

mutations in the Ptch1 gene on chromosome 9 are responsible for ligand-independent activation of the Shh pathway that predisposes the patients to high incidence of basal cell carcinoma (BCC), medulloblastoma, and rhabdomyosarcoma (Scales and Sauvage 2009). Ligand-independent activation of Shh pathway is also observed in almost all cases of sporadic BCC, either through tumor-suppressor gene Ptch1 inactivating mutations and/or loss of heterozygosity (>85%) or through activating mutation in Smo receptor (~10%), generating SmoM2 protein (Trp535Leu) (Reifenberger et al. 2005). Finally, inappropriate Shh pathway activation is observed in about one third of all medulloblastomas due to sporadic mutations in either Ptch1 or SuFu or Gli1 up-regulation (>30%) and in sporadic rhabdomyosarcomas where it has been described the loss of heterozygosity of the two negative regulators Ptch1 and Sufu (Tostar et al. 2006; Taylor et al. 2002).

Type II Sonic Hedgehog signaling: ligand dependent (autocrine mechanism).

Ligand dependent activation has been described in many tumors such as lung, breast, pancreatic, gastric, prostate, melanoma and colorectal cancers. These tumors are able to produce and respond to Shh ligand through both auto- or juxtacrine mechanisms where Shh ligand is produced and acts on the same tumor cells (or neighboring tumor cells) to stimulate proliferation and/or survival leading to tumor growth (**Fig.2B**) (Scales and Sauvage 2009).

Type III Sonic Hedgehog signaling: ligand dependent (paracrine mechanism).

In diffrent cancer models the tumor cells have been described to overproduce the Shh ligand that can stimulate stroma cells near the tumor in a paracrine manner. Shh ligand secreted by cancer cells acts on stroma cells leading to Shh pathway activation in the tumor microenvironment (**Fig.2C**). Upon Shh target genes transcription, the mesenchymal cells produce growth and survival signals that are feeded back to the tumor cells such as Insulin-like growth factor (IGF), Wnt and VEGF enhancing cancer progression and aggressiveness (Gupta et al. 2010). Tumor growth is then indirectly supported by mechanisms originated in the stroma cells (Heretsch et al. 2010). Therefore, paracrine ligand dependent Shh signaling activation has been described to mediate tumor maintenance and growth more than tumor initiation. A variant of this Shh signaling is the "reverse paracrine signaling" (**Fig.2D**) where the Shh ligand is secreted from the stroma and is received by the tumor cells. The reverse paracrine signaling is typical of hematological malignancies such as leukemia or multiple myeloma and B-cell lymphoma where stromal Shh is able to supply the appropriate microenvironment for tumor growth (Scales and de Sauvage 2009) and at the same time Shh ligand secreted from bone marrow stroma up-regulates anti-apoptotic factors, such as Bcl2, supporting cancerous B cells survival (Hegde et al. 2008).

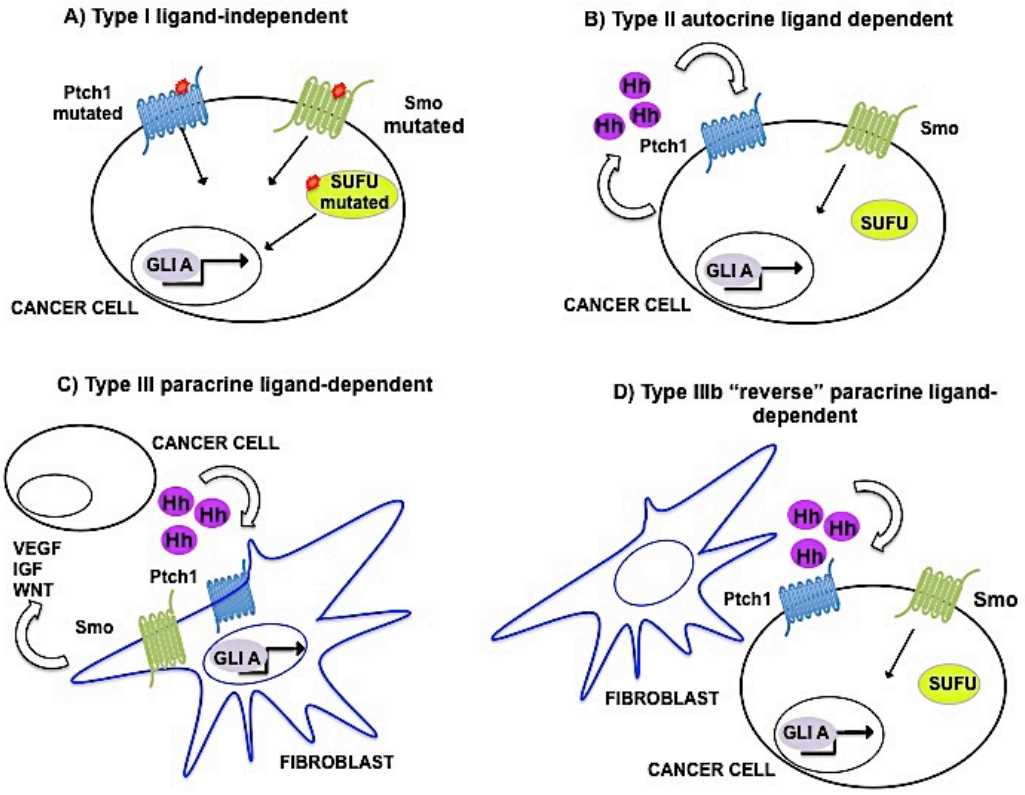


Figure 2. Mechanisms of Hedgehog signaling activation in cancer. A) Shh pathway is constitutively active, even in the absence of Shh ligands, due to inactivating mutations in Ptch1 receptor or in the negative regulator Sufu or due to activating mutations in Smo receptor. **B)** Cancer cells both produce and respond to Shh ligand leading to cell-autonomous signaling activation. **C)** Cancer cells produce and secrete Shh ligand which is received by stromal cells that then activate the pathway. In turn, stromal cells produce other growth or survival signals to the cancer cells. **D)** Stromal cells secrete Shh ligand that leads to Shh pathway activation in cancer cells. (Modified from Heretsch et al. 2010)

Several evidences describe the Hedgehog pathway as an important regulator of cancer-stroma interaction (Olive et al. 2009). Shh ligand, produced from pancreatic cancer cells and acting on pancreatic stromal cells, is able to activate the Hedgehog pathway in pancreatic stroma promoting the transcription of downstream genes. Shh signaling has been demonstrated to be involved in the regulation of the pancreatic tumor microenvironment, tumor development promotion and metastasis (Li et al. 2014). It has also been demonstrated an important role of Shh signaling in the stromal response to Shh ligand in the pancreatic ductal adenocarcinoma (PDA) formation. Lee and coworker (2014) have recently shown that Shh signaling inhibition in PDA mouse models accelerates progression of oncogenic Kras-driven disease, while pharmacologic inhibition of Shh pathway induced an alteration in the balance between stromal

and epithelial elements reducing desmoplastic reaction and promoting tumor growth. Shh signaling has been shown to be also involved in human tissues injury and inflammation where Shh ligand, secreted from injured or damaged cells, acts on stromal microenvironment (fibroblasts, immune cells and resident quiescent cells) to activate Shh signaling pathway.

Cancer stem cells (CSC) have the ability to both generate additional stem cells (self-renewal) and differentiate (multipotency), supporting tumor growth and propagation. Shh signaling pathway is involved in the CSC self-renewal regulation in different models of cancer such as breast, glioma and multiple myeloma (Theunissen and de Sauvage 2009). The Shh ligand can be produced either by CSCs or by the surrounding stroma and activates Shh signaling in self-renewing CSCs (**Fig.3**). Moreover, Shh signaling activation leads to CSC differentiation into cancer cells forming the bulk of the tumor (Gupta et al. 2010). Conventional chemotherapy and radiation are typically ineffective to fight CSCs that have been hypothesized as the cause of cancer relapse after treatment with conventional therapies. Shh signaling pathway activation in several types of CSCs allows to consider Shh inhibition as a promising therapeutic target to fight CSCs (Scales and de Sauvage 2009).

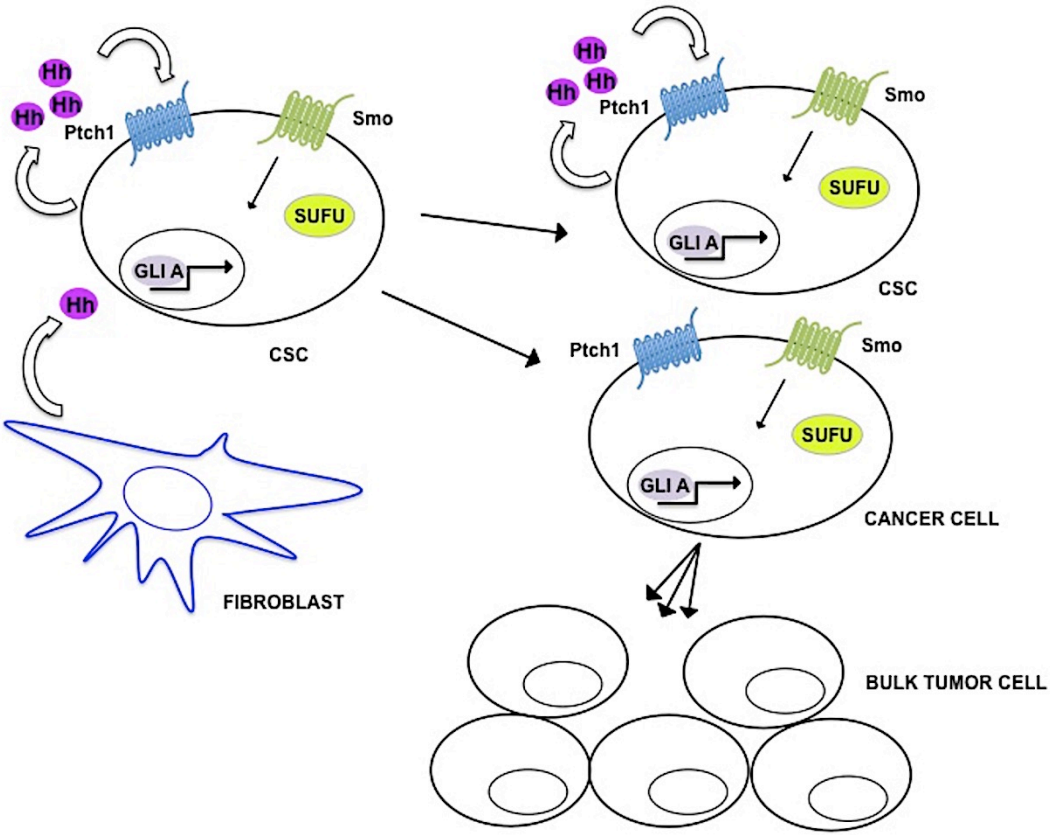


Figure 3. Hedgehog pathway in cancer stem cells. Cancer stem cells produce their own Shh ligand, or receive it from stromal cells, activating Shh signaling that stimulates and supports stem cell self-renewing. As well as CSC differentiation into cancer cells that form the bulk of tumor. (Modified from Gupta et al. 2010).

1.5 Hedgehog pathway inhibitors (HPIs)

The great involvement of aberrant Shh signaling pathway in several cancers makes it an attractive anticancer drug target. Several small molecular inhibitors, either natural products or synthetic compounds, have been developed in order to target different regulative elements of Shh pathway.

1.5.1 Smoothened inhibitors

The steroidal alkaloid cyclopamine was isolated from the *Veratrum californicum* and identified as a teratogen compound responsible for severe craniofacial defects such as cyclopia and brain malformations (Keeler and Binns 1968). It was afterwards proven that cyclopamine teratogenic effects resulted from its ability to inhibit Shh pathway. Cyclopamine binds directly to Smo protein on the heptahelical domain that represents its specific binding site. Cyclopamine binding influences the Smo protein conformation leading to the inhibition of Smo activity, even in the presence of Shh ligand (Chen et al. 2002). Therefore this drug represents a valuable strategy for the treatment of tumors characterized by Smo hyperactivation due to Ptch loss of function mutations or Smo gain of function mutations and has been provided for the treatment of Basal cell carcinoma (BCC) through the use of a cream formulation applied locally. BCCs treated with cyclopamine have shown a substantial regression (Tabs and Avci 2004). However, cyclopamine displays low affinity for the Smo receptor, poor oral bioavailability, low metabolic stability, and suboptimal pharmacokinetics therefore, its pharmacological and physicochemical properties do not make it an ideal drug (Tremblay et al. 2008). For this reason, more potent derivatives of cyclopamine have been synthesized. One of the first semi-synthetic cyclopamine-based inhibitors is KAAD-cyclopamine (3-keto-N-(aminocaproyl-dihydrocinnamoyl cyclopamine) showing higher potency and reduced cytotoxicity compared to its precursor (Heretsch et al. 2010). The small molecule Hedgehog inhibitor IPI-269609 (Infinity Pharmaceuticals), another cyclopamine derivative, retains the potency of cyclopamine but shows high solubility and acid stability. It resembles the properties of cyclopamine to block Hedgehog pathway in particular in pancreatic cancer cells *in vitro,* impairing cell motility and colony formation, and inhibiting metastasis formation *in vivo* (Feldmann et al. 2008). IPI-269609 could therefore be subjected to future clinical testing for use in humans. A second generation of cyclopamine analogue is represented by Saridegib (IPI-926), characterized by improved pharmaceutical properties and a favorable pharmacokinetic with respect to cyclopamine and IPI-269609. IPI-926 has progressed into human clinical trials, in particular, it is in

clinical trials phase 1b/2 for metastatic pancreatic cancer (in combination with Gemcitabine chemotherapy) and in phase 1 (in combination with monoclonal antibody Cetuximab) for recurrent head and neck cancer. IPI-926 is also involved in advanced phase 2clinical trials, in particular for ly (MF) and for conventional chondrosarcoma (www.clinicaltrials.gov).

The Shh inhibitor Vismodegib (GDC-0449 Curis-Genetech) has been identified through a high-throughput screening of a library of small-molecule compounds. GDC-0449 is a potent, selective and orally bioavailable Hedgehog antagonist characterized by favorable pharmaceutical properties (Rudin et al. 2009). Although it is structurally unrelated to cyclopamine and cyclopamine analogues, GDC-0449 is able to bind to Smo with high specificity and affinity leading to an effective Shh signaling pathway down-regulation (Robarge et al. 2009). Vismodegib has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of either metastatic or locally advanced basal cell carcinoma in patients unsuitable for surgical or radiation therapy (Rudin 2012). GDC-0449 has entered in phase II trial for metastatic colorectal cancer, ovarian cancer, adult brain tumors and childhood medulloblastoma (www.clinicaltrials.gov). Nonetheless, acquired resistance to GDC-0449 has been observed in patients with medulloblastomas. The D473H mutation has been in fact identified in the Smo protein upon Vismodegib treatment (Yauch et al. 2009). Such mutation results in an amino acid substitution from an aspartic acid to a histidine at the position 473 at the boundary between the 6th trans-membrane domain and the extracellular domain of Smo protein (www.mycancergenome.org). Acquired resistance events due to additional Smo mutations and variants have been found also in BCC patients treated with Vismodegib and in a phase I clinical trials BCC patients treated with Saridegib (IPI-926) (Jimeno et al. 2013). Another synthetic, effective, small-molecule Smo antagonist is the BMS-833923 (Bristol Myers Squibb). It has entered in phase II trial, in combination with Dasatinib (BMS-354825) for chronic myeloid leukemia and in phase I trial for BCC, Basal cell nevoid Syndrome, small cell lung carcinoma and stomach neoplasm (www.clinicaltrials.gov). HhAntag is another small-molecule Smo-binding Shh pathway inhibitor. In medulloblastoma in vivo models HhAntag's ability to block tumor cell proliferation and stimulate apoptosis has been demonstrated. It is also able to inhibit growth of some Shh-dependent paracrine tumors, although off-target effects have been observed at high concentrations (Romer et al. 2004). It has recently been shown in vitro that HhAntag is able to block the process of chondrogenesis, a characteristic of the pediatric disorder Hereditary Multiple Exostoses, by blocking the Shh pathway which is one of its major regulators (Mundy et al. 2015). Cur-61414 is a topical synthetic Smo inhibitor. In BCC ex-vivo mouse model this inhibitor has proven ability to reduce cell proliferation and induce apoptotic death (Scales and de Sauvage 2009). It has entered phase I clinical trials for sporadic BCC, but the trial was interrupted due to failure possibly related of the molecular formulation which did not properly penetrate

human skin. Recently a new class of Smo inhibitor has been identified; SMANT (Smo Mutant ANTagonist), that exhibit an equivalent activity in inhibiting wild-type Smo and oncogenic mutated form of Smo (SmoM2 Trp535Leu). This creates the possibility to develop new therapeutic strategies for the treatment of Smo inhibitor-resistant tumors characterized by Smo gain-of-function mutations. In fact, acquired resistance to Smo antagonist represents one of the most important obstacles in the treatment of Shh signaling pathway dependent cancer. According to recent evidences, cancer cells can acquire resistance to Smo inhibitors also through Smo-independent hyper-activation or mutation in another Shh signaling downstream component (Infante et al. 2015). Preclinical and clinical trials have shown that Smo resistance can be caused for instance by Gli protein amplification, up-regulation of non-canonical Gli signaling activation or increase of the expression of ATP-binding cassette transporter (ABCs) that increase cellular clearance of the drug (Yauch et al. 2009; Rudin et al. 2009).

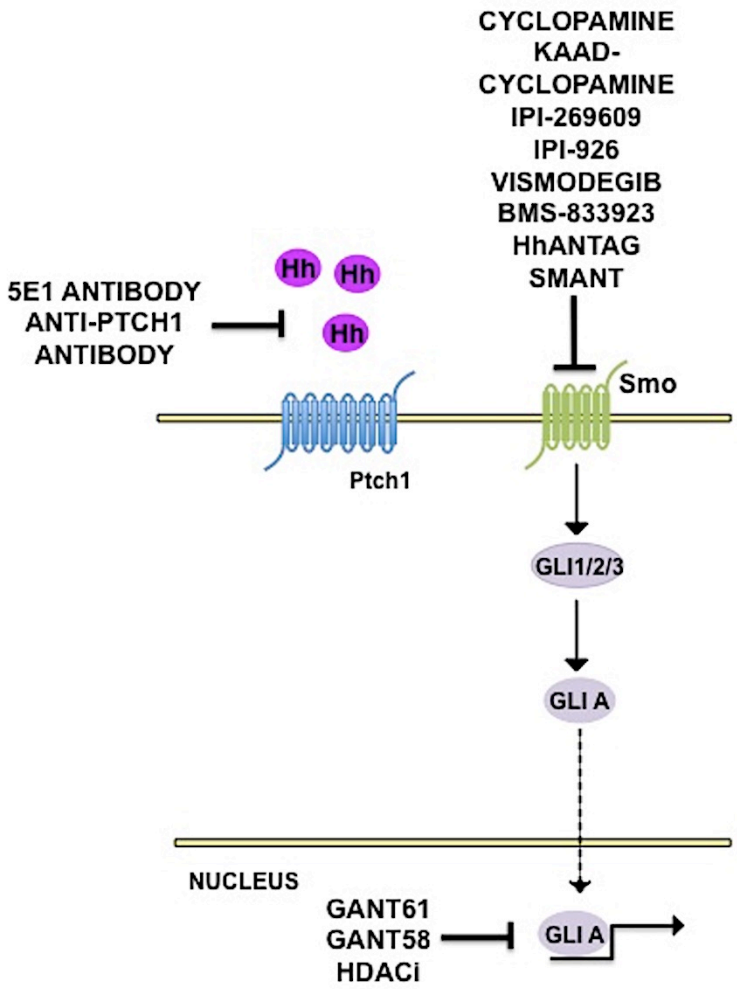


Figure 4. Hedgehog pathway molecular targets. Schematic representation of the targets of several Shh inhibitors.

1.5.2 Upstream Smoothened inhibitors

Shh pathway inhibitors may also prevent the ligand binding to its receptor Ptch. Hh-blocking antibody 5E1 is a monoclonal antibody used in the study of Shh signaling pathway involvement in both embryonic development and tumorigenesis.

It is able to block Shh signaling by inhibiting the binding of all three mammalian Hh ligands (Sonic, Indian and Desert Hedgehog) to Ptch receptor (Maun et al. 2010). 5E1 antibody has been employed in in vivo pancreatic cancer models proving a decreased tumor volume and a significant reduction of desmoplasia (Bailey et al. 2008). Antibodies against Ptch1 receptor were also generated with the aim to inhibit the Shh pathway. Anti-patched1 antibody acts against an oligo-peptide within the amino acid sequence of the receptor involved in ligand binding. A suppressive effect on Shh signaling has been shown in in vitro models of pancreatic cancer resulting in a reduction of cell proliferation (Nakamura et al. 2007).

1.5.3 Downstream Smoothened inhibitors

For tumors characterized by mutations downstream of the Smo receptor, such as inactivating mutation of SuFu or Gli1 amplification, treatment with Smo agonists or with upstream Smo inhibitors is ineffective. In order to block downstream Shh signaling, small molecules that are able to inhibit the zinc-finger transcription factors Gli have been identified. Gli antagonists can be classified according to their mechanisms of action in: direct Gli antagonists, that are able to block Gli mediated gene transcription, interacting directly with the zinc finger proteins; indirect Gli antagonists, that act controlling Gli activation mechanisms such as proteolytic degradation and post-translational modifications (Infante et al. 2015). GANT61 (Gli-ANTagonist) and GANT58 are two direct Gli antagonists, structurally different, but both capable of interfering with Gli1 and Gli2-mediated transcription (Lauth et al 2007). Both Gli inhibitors act at the nuclear level to block Gli induced transcription, in particular GANT61 is able to destabilize DNA-Gli complex binding to the 5-zinc finger Gli protein, between zinc finger 2 and 3, at the amino acid sites E119 and E167. The GANT61 specific binding sequence is conserved between Gli1 and Gli2 zinc finger proteins, therefore the drug is able to bind both Gli zinc finger proteins (Agyeman et al. 2014).

Gli zinc finger proteins can be activated by oncogenic pathways that bypass the Shh signaling pathway. For instance KRAS and BRAF genes, which are highly recurrent in colon cancers, activate a ligand-independent pathway, by interfering with the PKA-negative regulation and increasing the Gli1 and Gli2 nuclear localization and transcriptional activation, consequently promoting cellular proliferation and tumor progression (Mazumdar et al. 2011; Rovida and Stecca 2015). According to recent evidences, GANT61 inhibitor is able to

inhibit Gli1 and Gli2 mediated transcription even in tumors where the zinc finger proteins activation is mediated by Shh-independent pathways (Lauth et al. 2007). GANTs inhibitors' effects on Shh pathway has been demonstrated in *in vitro* models, where both GANT58 and GANT61 lead to inhibition of the tumor cell proliferation and tumor cell growth, and in *in vivo* human prostate cancer xenograft models where they suppress tumor growth (Lauth et al. 2007). The HDAC (Histone Deacetylases) inhibitors (HDACis) represent another class of Shh antagonists that act as indirect Gli antagonists preventing Gli1 and Gli2 transcriptional activity. Gli1 and Gli2 are acetylated proteins that need a HDAC-mediated deacetylation for the transcriptional activation. By inhibiting deacetylation process of the zinc finger proteins, HDACis prevent the gene transcriptional activation and induce Gli1 and Gli2 hyper-acetylation (Coni et al. 2013). Vorinostat (SAHA), Romidepsin and Belinostat are three HDACis that have been approved by the FDA for the treatment of peripheral T cell lymphoma (Mottamal et al. 2015). Vorinostat is also in the phase 1 trial (in combination with radiation therapies) for treatment of patients with brain metastasis and (in combination with hydroxychloroquine, HCQ) for treatment of malignant solid tumors. Romidepsin is in the phase 1 trial for solid tumors, multiple myeloma, Her2-negative breast cancers and triple negative cancers treatment. Moreover, Belinostat is in phase 1 trial for ovarian cancer (plus Carboplatin and/or Paclitaxel) and solid tumors and in the phase II for the glioblastoma multiforme (GBM) (www.clinicaltrials.gov).

1.6 Role of Sonic Hedgehog in thyroid

1.6.1 Sonic Hedgehog in thyroid development

The sonic hedgehog pathway is involved in the regulation of different developmental processes in vertebrates such as left-right axis determination of the embryo, tissue patterning and organogenesis (McMahon et al. 2003). Moreover Sonic hedgehog has been found to have an important role in proper thyroid development. Shh signaling regulates the thyroid symmetric bilobation in late organogenesis and represses the improper thyroid differentiation in nonthyroid embryonic tissues (Fagman et al.2004). Studies in Shh -/- mice models have shown the importance of Sonic Hedgheog pathway in thyroid embryonic development. Shh-deficient mice display a correct development of thyroid during the early phases of embryogenesis, but, in the late phase, the thyroid gland fails the separation into two distinct lobes and appears as a single tissue mass displaced unilaterally on the left side of the midline (Fagman et al. 2004). Despite the important thyroid dysgenesis, the thyrocytes development takes place normally forming polarized epithelium and follicles, although an ectopic thyrocytes development outside the thyroid tissue, in particular in the primitive respiratory epithelium, has been observed in Shh-/- mice. Thyroid hemiagenesis has been described in several genetic syndromes, such as in Williams syndrome, a complex genetic disorder characterized by developmental delays, cardiovascular disease and learning disabilities caused by deletion at 7q11.23 chromosome (Nickerson et al. 1995). Genes interacting with Shh signaling, such as Frizzled (G protein-coupled receptor involved in the Wnt signaling pathway), have been linked to thys syndrome because mapped in the deleted region 7q11.23 (Wang et al.1997), supporting the connection between thyroid development and Shh pathway activity.

1.6.2 Sonic Hedgehog in thyroid tumors

The role of Sonic Hedgehog pathway has been studied and characterized also in thyroid tumors. Recent studies have shown a wide expression of Shh pathway components such as Ptch and Smo receptors and the transcription factor Gli1 in PTC, ATC and in benign follicular thyroid adenoma (FTA) suggesting that the Shh pathway activation occurs at an early phase of thyroid tumor development. The expression of Shh pathway components has been also found in ATC suggesting that an aberrant activation of this pathway can be involved in anaplastic thyroid transformation (Hinterseher et al. 2013). Finally, an increased expression of the Shh signaling component in medullary thyroid cancer with respect to normal thyroid tissue has been demonstrated (Bohinc et al. 2013). Nonetheless, the activation of the Shh pathway does not seem to be causative of thyroid neoplastic transformation, but it rather seems to promote thyroid tumorigenesis and progression in collaboration with other oncogenic pathways

(Xu. et al 2012). As previously mentioned, a cross-talk between the MAPK pathway and the Shh signaling has been demonstrated in pancreatic tumorigenesis. By suppressing the Gli protein degradation process, the oncogenic KRAS ^{G12V} (Gly12Asp), through the RAF/MEK/ERK pathway, leads to increased amount of Gli1 and increases its transcriptional activity. This ligand-independent activation has been correlated to progression of pancreatic ductal adenoma to carcinoma (Ji et al 2007). Thyroid neoplasms are frequently charaterized by genetic alterations that lead to the activation of the MAPK signaling pathway however, it has not been described yet a connection between MAPK pathway and Shh signaling activation in thyroid neoplastic transformation. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is a transcription factor involved in numerous processes such as proliferation, apoptosis and inflammation in both development and tumorigenesis (Karin et al 2002). NF-kB involvement in thyroid cancer has also been demonstrated (Pacifico and Leonardi 2010). Several NF-kB binding sites have been identified at the position +139 of the human Shh promoter region (Kasperczyk et al. 2009), therefore NF-kB pathway activation could contribute to increased Shh ligand expression levels that can lead to Shh pathway activation (in autocrine or paracrine manner) in thyroid cancers. The involvement of p63 protein in the Shh expression regulation has also been demonstrated. p63 is an homologue of the tumor suppressor protein p53 involved in development and tumorigenesis. The p63 gene encodes for six different isoforms, TAp63 (α,β,γ) and ΔNp63 (α,β,γ), with or without transactivation domain respectively. It has been demonstrated that TAp63γ isoform is able to bind the region within the Shh promoter, located between nucleotides -228 and -102, inducing Shh transactivation (Caserta et al. 2006). P63 seems to be expressed in PTC but not in normal thyroid tissue (Unger et al. 2003). Therefore, p63 protein could also be implicated in the regulation of the Shh signaling pathway in thyroid tumorigenesis.

Finally, recent evidences suggest the Shh pathway involvement in the promotion of thyroid CSC self-renewal in anaplastic cell lines. It is known that Gli1 transcription factor activation induces the expression of the zinc finger protein Snail (Heiden et al. 2014). Snail is a transcription factor implicated in the differentiation of epithelial cells into mesenchymal cells (epithelial - mesenchymal transition, EMT) during embryonic development (Cano et al. 2000). Normal thyroid epithelial cells do not express the Snail transcription factor that instead it is widely expressed in thyroid tumor cell lines and tissues (Hardy et al. 2007). Recent studies have demonstrated that the Shh pathway inhibition, in anaplastic thyroid cancer cells, leads to decreased Snail expression, decreased *in vitro* thyrosphere formation and decreased aldehyde dehydrogenase (ALDH) positive CSCs (Heiden et al. 2014).

2.0 AIM OF THE STUDY

Sonic Hedgehog pathway has been increasingly studied due to its involvement in different human cancers. Several reports suggest the aberrant activation of Shh signaling as an important event during neoplastic transformation. A number of proliferative and oncogenic pathways have been described to cross-interact with Shh pathway in cancer (Pandolfi and Stecca 2015). Moreover, Shh signaling has been demonstrated to be one of the main signaling pathways activated in tumor stroma and involved in tumor-stroma interaction.

Recent evidences suggest a role of Shh signal transduction in thyroid development and tumorigenesis, but the mechanisms of this activation have not been described yet. Here we have studied the role of Shh pathway in thyroid cancer and its involvement in tumor interaction with microenvironment. To this aim we have:

1) Analyzed the expression and activation of Shh signaling components in thyroid tumors and tumor-derived cell lines;

2) Studied the molecular mechanisms regulating Shh pathway in thyroid cancer;

3) Investigated the Shh signaling involvement in regulating tumor stroma interaction in aggressive thyroid cancer (ATC).

3.0 MATERIALS AND METHODS

3.1 Compounds

For *in vitro* experiments, cyclopamine hydrate was purchased by Sigma-Aldrich (St. Louis, MO, USA). Cyclopamine was dissolved in dimethyl sulfoxide (DMSO) at concentration of 3mM and stored at -80°C. Vandetanib (ZD6474) and Vemurafenib (PLX4032) were dissolved in DMSO at a concentration of 50 mM and stored at -80°C. Azacytidine (Sigma Aldrich) was used at a concentration of 4 μM for 48 hours.

3.2 Cell lines

TPC1 cells were originally obtained by M. Nagao (Carcinogenesis Division, National Cancer Center Research Institute, Tokyo, Japan). SW1736 were obtained by N.E. Heldin (University Hospital, S-751 85 Uppsala, Swden). BCPAP were obtained by the primary source (N. Fabien, CNRS URA 1454, University of Medecine Lyon-Sud, Oullins, France). 8505C, CAL62 anaplastic carcinoma cells were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) (Salerno et al. 2010). OCUT cells were provided by N. Onoda (Osaka University of Medicine, Osaka, Japan) in 2005. NTHY are normal human thyrocytes immortalized by the Large T of SV40 and were obtained from the European Tissue Culture collection. HEK293 cells were from American Type Culture Collection (ATCC). All cells grown in either Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium supplemented with 10% fetal bovine serum. All media were supplemented with 2 mM L- glutamine and 100 units/ml penicillin-streptomycin (GIBCO). The Fischer rat-derived differentiated thyroid follicular cell line PC Cl 3 was grown in Coon's modified Ham F12 medium supplemented with 5% calf serum and a mixture of six hormones (6H): thyrotropin (10mU/ml), hydrocortisone (10nM), insulin (10μg/ml), apo-transferrin (5μg/ml), somatostatin (10ng/ml), and glycyl-histidyl-lysine (10ng/ml) (Sigma-Aldrich). PC adoptively expressing several oncogenes have been cultured in the same medium as PC but without the 6H.

3.3 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 Prof. 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. Informed consent was obtained from the patients and the study was approved by the institutional review board committee. Tumor size, extra-thyroid 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.

3.4 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 fore 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, USA). As a negative control, tissue slides were incubated with isotype-matched IgG1 control antibodies. The percentage of positive cells for Gli1 staining was evaluated.

3.5 Immunoblotting

Protein lysates were prepared according to standard procedures. Briefly, cells were harvested in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10 μg of aprotinin/ml, 10 μg of leupeptin/ml) and clarified by centrifugation at 10,000 x*g*. Nuclear proteins extraction was performed using NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit (#78835) (Thermo scientific Rockford, USA) according to manufacturer's instructions. Protein concentration was estimated with a modified Bradford assay (Bio-Rad) and lysates were subjected to SDS PAGE. Membranes were probed with the indicated antibodies. Immune complexes were revealed by an enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech). Signal intensity was quantified with the Phosphorimager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software.

3.6 Antibodies

Anti Shh (#2207), Anti Gli1 (#2553) and Anti Sufu (#2520) are rabbit monoclonal antibodies and were from Cell Signaling Technology. Anti Smo (E-5) sc-166685 (#C1315), mouse monoclonal antibody and Anti Gli2 (H-300) sc-28674 (#K2013), rabbit polyclonal antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibody anti-Parp (#9542) was from Cell Signaling Technology. Monoclonal anti-tubulin (#T9026) was from Sigma-Aldrich (St Louis, MO, USA). Secondary antibodies coupled to horseradish peroxidase were from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

3.7 Quantitative real-time PCR

Total RNA was isolated with the RNeasy Kit (Qiagen, Crawley, West Sussex, UK). One μg of RNA from each sample was reverse-transcribed with the QuantiTect® Reverse Transcription (Qiagen). PCR reactions were performed in triplicate and fold changes were calculated with the formula: 2-(sample 1 ΔCt - sample 2 ΔCt), where ΔCt is the difference between the amplification fluorescent thresholds of the mRNA of interest and the mRNA of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as an internal reference.

3.8 DNA constructs and Reporter assay

pGL3-Gli-Luc was obtained by PCR-cloning eight copies of the Gli binding element into the pGL3 enhancer vector purchased from Promega. Luciferase activities present in cellular lysates were assayed using the Dual-Luciferase Reporter System (Promega, Madison WI, USA). In all cases, total amount of transfected plasmid DNA was normalized by adding *Renilla* luciferase reporter gene. Light emission was quantitated using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). PSec-Shh construct has been previously described (Castellone et al. 2015). Data were represented as luciferase activity present in each sample and the values plotted were the average ± SEM of triplicate samples for typical experiments, which were repeated at least 3-5 times with nearly identical results.

3.9 Cell transfection and generation of stable cells

Gli1-specific shRNA expression vector (#TG312756) and a scrambled control vector (#TR30013) were purchased from OriGene Technologies, (Inc.9620 Medical Center Drive, Suite 200 Rockville). Experimental procedures were done as follows: the day before transfection, cells were plated in 35-mm dishes at 40% of confluence in DMEM supplemented with 10% FBS without antibiotics. Two μg of sh-Gli1 and control vector were transfected using FuGENE HD Transfection (Promega) reagent according to manufacturer's instructions. Seventy-two hours after transfection, cell medium was supplemented with puromycin (Sigma-Aldrich) at final concentration of 1μg/μl for 14 days. Stably transfected cells were screened by RT-PCR as previously described.

3.10 Cell proliferation assay

$3x10^4$ cells were plated in 60-mm dishes. Cells were kept in RPMI 1640 or DMEM supplemented with 10% fetal calf serum. The day after plating, compounds or vehicle were added. Cells were counted in triplicate every 24

hours. To estimate IC50 value, cells were counted after 72 hours.

3.11 Wound healing assay

A wound was induced on the confluent monolayer cells by scraping a gap using a micropipette tip. Photographs were taken at 10X magnification using phase-contrast microscopy immediately after wound incision and up to 24 hours later.

3.12 Matrigel 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 800Xg for 10 minutes. The cell suspension ($2X10^5$ 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.). Stromal cells ($5X10^5$ cells/well) were plated on the bottom of wells. The lower chamber was filled with complete medium. Cell dishes were incubated at 37°C in 5% CO_2 and 95% air for 48 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.

3.13 Spheroid-forming assay

50 cells per well were plated on ultra-low-attachment 96-well (Corning Incorporated, Painted Post, NY, USA) in low serum DMEM medium. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Spheroids were photographed after 7 and 14 days.

3.14 Statistical analysis

Cell growth comparisons were done applying the Unpaired Student's t test (Instat software, Graphpad Software Inc). All P values were two- sided, and differences were considered statistically significant at $P < 0.01$. IC50 values were calculated through a curve fitting analysis from last day values using PRISM software (Graphpad). P values were statistically significant at $P < 0.01$.

4.0 RESULTS

4.1 Sonic Hedgehog pathway expression in thyroid cancer cells

Recent evidences suggest a role of the Shh pathway in thyroid tumorigenesis (Hinterseher et al. 2013; Bohinc et al. 2013). In order to characterize the expression levels of Shh pathway molecules in a panel of papillary (TPC1, BCPAP) and anaplastic (SW1736, 8505C, CAL62, OCUT) thyroid cancer cells compared to immortalized NTHY cell line (derived from non-tumoral tissues), we performed immunoblotting experiments.

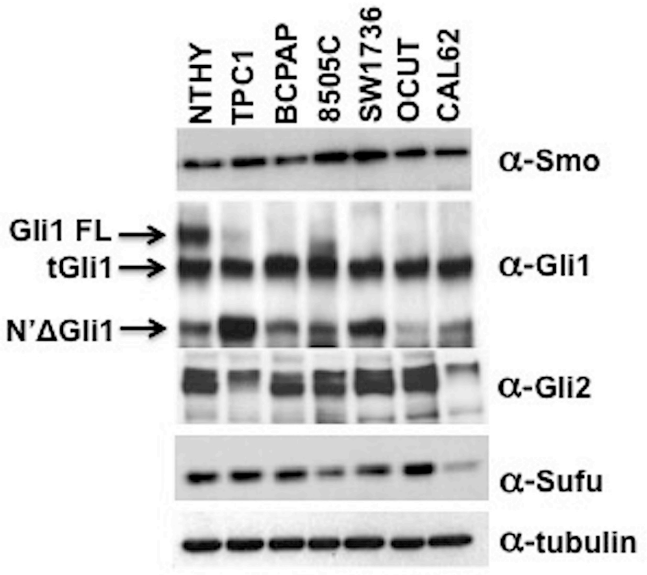


Figure 5. Shh pathway components are expressed in thyroid cell lines. Total cell lysates (50μg) were analyzed with anti-Smo, anti-Gli1, anti-Gli2 and anti-Sufu antibodies. Total tubulin level was used for normalization.

As shown in figure 5, the Smo receptor was expressed in all thyroid cell lines and it was slightly increased in TPC1, 8505C and SW1736 cancer cells. The Gli1 transcript can undergo alternative splicing producing a truncated Gli1 variant (tGli1 ~155kDa) lacking the exon 3 and part of exon 4 (41aa deletion), and therefore lacking the Sufu binding domain. N'ΔGli1 isoform (~130kDa) may also be generated as a result of a post-translational N-terminal truncation of Gli1 full length (FL) lacking inhibitory Sufu binding domain and degron domain (Carpenter and Lo 2012). Both these variants have been described to be particularly expressed and active in cancer (Cao et al. 2012; Amable et al. 2014). Interestingly, when analyzing the Gli1 expression levels we found differences in the levels of the different isoforms; in particular: Gli1 FL isoform (160kDa) seems to be highly expressed in NTHY cell line and modestly expressed in

TPC1 cells while all the other cancer cell lines do not express the FL isoform but instead they have higher relative levels of the tGli1 isoform compared to NTHY cells; N'ΔGli1 isoform levels were higher in TPC1 and SW1736 cells. Gli2 transcription factor was expressed in all thyroid cell lines, showing lower levels in TPC1 and CAL62 cells. Finally, Sufu inhibitor was expressed in a similar manner in all thyroid cells with the exception of CAL62, where it was down-regulated. As we were not able to find an antibody that would recognize the Ptch1 receptor in our cells, we performed quantitative RT-PCR (Q-RT-PCR) to measure the Ptch1 receptor expression levels in all thyroid cancer cell lines and NTHY cells. Ptch1 was slightly up-regulated in 8505C, SW1736 and OCUT cells with respect to NTHY cells (it is known that Ptch1 is a transcriptional target of Gli1). Interstingly, Ptch1 expression levels were clearly down-regulated in BCPAP cell line (**Fig.6)**.

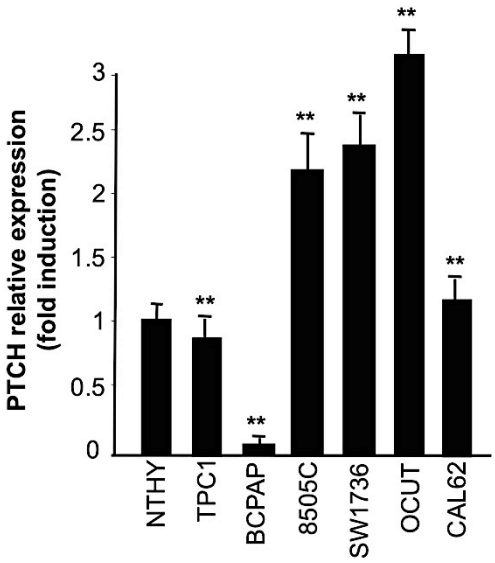


Figure 6. Ptch1 expression levels in thyroid cells. Q-RT-PCR of Ptch1in thyroid cells. Error bars represent standard deviations of experimental triplicates.

We also analyzed Shh ligand expression in thyroid cancer cells and NTHY. Immunoblotting experiments did not show a detectable expression level of such protein, therefore we performed quantitative RT-PCR (Q-RT-PCR) demonstrating that Shh was expressed at very low levels (amplification was reached above 33 cycles) in all thyroid cell lines and slightly up-regulated SW1736 cells with respect to NTHY cells **(Fig.7)**.

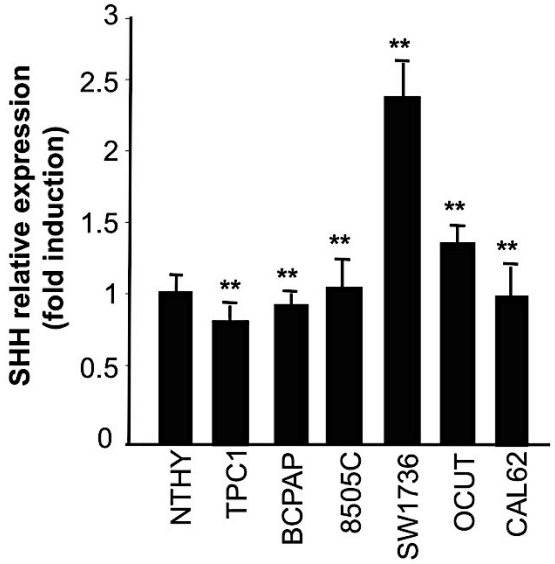


Figure 7. Shh expression levels in thyroid cells. Q-RT-PCR of Shh in thyroid cells. Error bars represent standard deviations of experimental triplicates.

4.2 Activation of Sonic Hedgehog pathway in thyroid cancer cells

In order to test the activation of the Shh pathway in thyroid cells, we transfected a reporter Gli-Luc plasmid (characterized by the luciferase cDNA under the control of 8 tandem Gli1-responsive elements) in a panel of cancer cell lines and control NTHY cells. As shown in figure 8, the Gli-Luc reporter is activated in all thyroid cancer cell lines but not in NTHY cells suggesting that the GLI transcriptional activation, marker of Shh pathway activity, takes place in thyroid cancer cells and not NTHY thyroid cells.

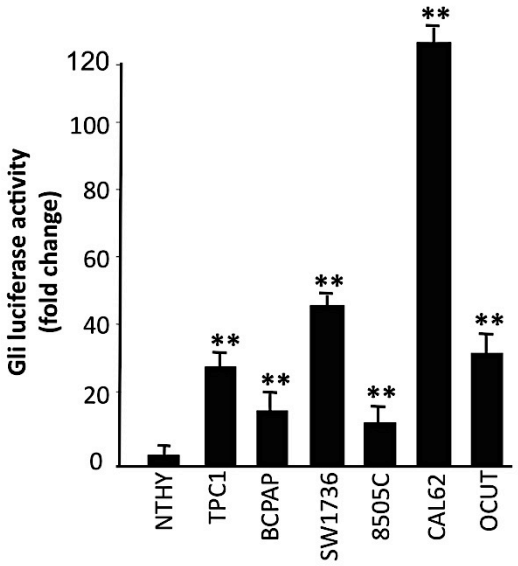


Figure 8. Shh pathway is active in thyroid cancer cells. Thyroid cells were transfected with Gli-Luc reporter (pGL3-Gli-Luc) and 48 hours after transcription Gli activation was measured by Luciferase assay. Error bars represent standard deviations of experimental triplicates.

To test whether the Gli transcriptional activation observed in our cells would depend on the Smo receptor, we performed a Gli-Luc assay upon treatment with the Smo antagonist cyclopamine (10uM) for 24h. Our results, shown in figure 9, demonstrate that in cancer cells and not in NTHY there is activation of Gli-Luc that is inhibited by cyclopamine. Moreover, cells were responding to stimulation with Shh ligand (produced in HEK293T cells upon transfection of a pSec-Shh plasmid).

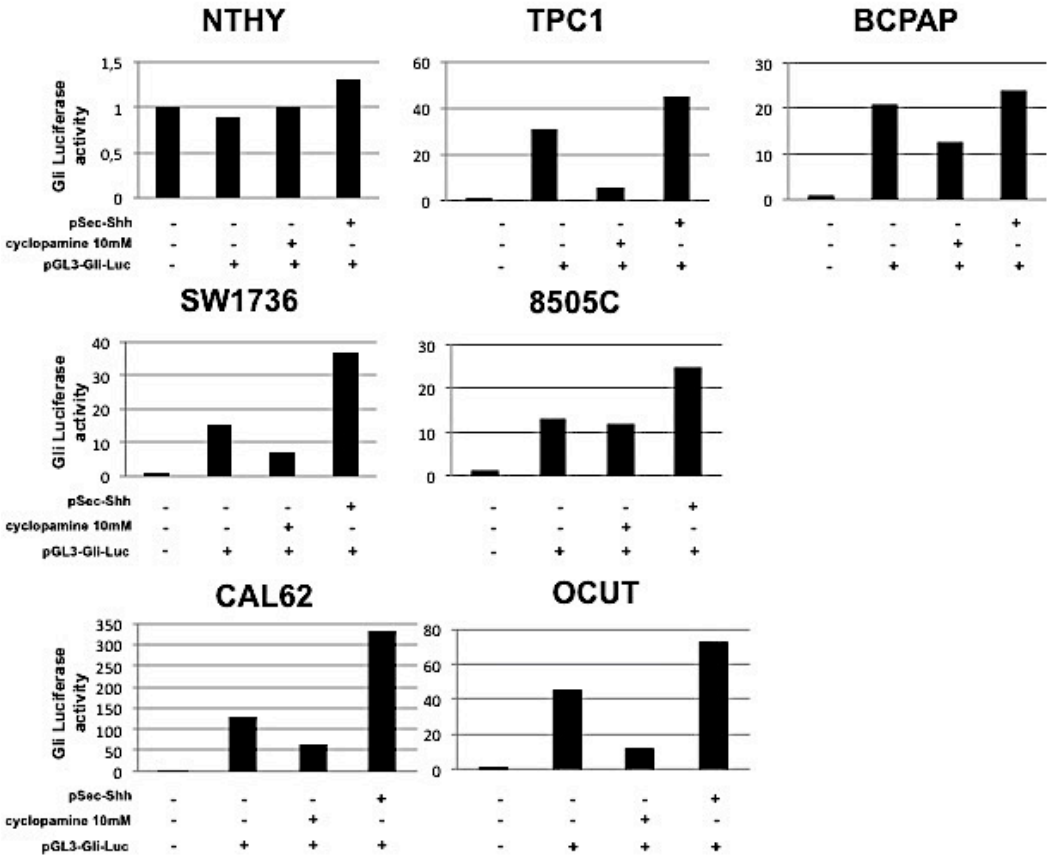


Figure 9. Shh pathway is active in thyroid cancer cells but it is inhibited by cyclopamine. Thyroid cells were transfected with Gli-Luc reporter (pGL3-Gli-Luc) and after 48 hours Gli transcription factors activation was measured by Luciferase assay. Thyroid cells treated with cyclopamine 10μM for 24h showed reduction of Gli transcription factors activation. Shh ligand stimulation was inducing reporter activation in cancer cells.

4.3 Up-regulation of Sonic Hedgehog pathway components in thyroid tumors

To study the expression of molecules of the Shh pathway in thyroid tumors, we evaluated Gli1 expression levels by immunohistochemistry in a panel of 66 human tissues including: 10 PTCs, 16 ATCs, 10 MTCs, 14 FTCs, 11 benign adenomas and 5 normal thyroids (NT). Normal thyroids were all negative for Gli1, while tumors were constantly positive, with a proportion of positive cells higher in ATC than in adenoma and FTC (**Fig.10** and **Table I**). Among PTC, PTC-TCV positivity was particularly high while in PTC-FV was lower and 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%) (data not shown).

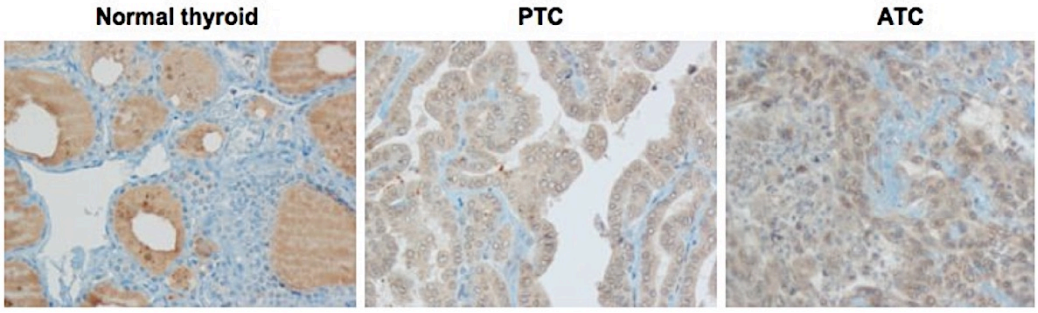


Figure 10. Gli1 is up-regulated in thyroid tumors. Representative histological sections from normal thyroid, PTC and ATC incubated with a mouse monoclonal anti-Gli1 antibody. ATC samples showed intense immunoreactivity for Gli1, PTC samples showed a mild expression of Gli1 while normal thyroid tissues were completely negative.

HISTOTYPE	Number of cases	Positive cases	% positive cells (mean)
PTC	10	9	45
ATC	16	16	69
MTC	10	10	66
FTC	14	10	46
Adenoma	11	8	42
Normal	5	0	

Table I Thyroid samples of different histotypes were stained for Gli1 expression. The majority of tumor samples resulted positive for Gli1 while all normal cases were negative. Average positive cells were calculated for each histotype.

To determine whether Gli1 up-regulation also occurred at the RNA level, we examined a set of normal, PTC and ATC tissues by quantitative RT-PCR. Furthermore, we measured the expression of Shh ligand and PTCH1 receptor (**Fig.11**). All components of the Shh pathway, though with significant variability among samples, were expressed in tumors at higher levels compared to normal tissues (mean of 5 normal thyroids).

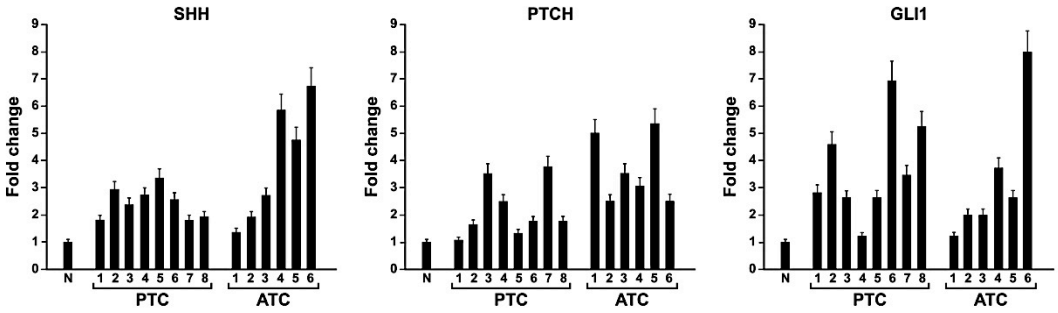


Figure 11. Shh signaling components are up-regulated in thyroid tumors. Q-RT-PCR of Shh, Ptch1 and Gli1 was performed in eight PTCs and six ATCs tumor samples compared to a pool of five normal thyroid tissues. The results are the average of three independent experiments. Error bars represent standard deviations of experimental triplicates.

4.4 Cyclopamine treatment reduces proliferation of thyroid cancer cells

As luciferase data suggested that cancer cells were sensitive to cyclopamine, we decided to measure cyclopamine-inibitory concentration 50 (IC50) for proliferative thyroid cancer cell lines and NTHY control cells by using increasing doses of the compound (**Fig.12**). Treatment of cancer cells with cyclopamine reduced proliferation with an IC50 ranging between 4.64 and 11.78μM while 20μM of cyclopamine did not reach IC50 in NTHY cells suggesting specificity of the drug for transformed cells.

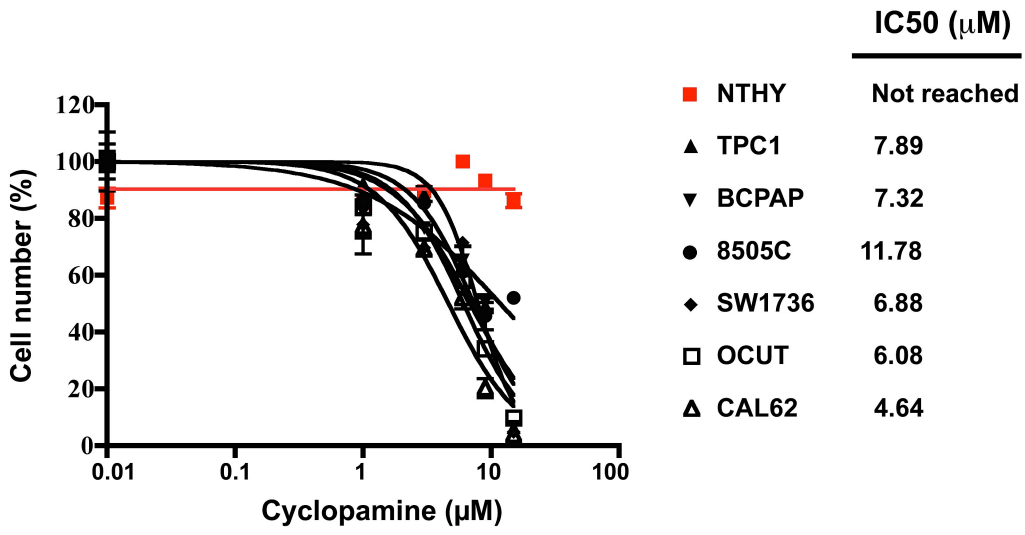


Figure 12. Thyroid cancer cells are sensitive to cyclopamine. Sensitivity of thyroid cells to cyclopamine was assessed by cell count. Thyroid cells were treated with different doses of cyclopamine (1-5-10-20μM). After 72 hours cells were counted in triplicate.

To test the effect of cyclopamine on thyroid cancer cells viability and proliferation we performed a growth curve by treating the cells for 72h. As shown in figure 13, 72h of cyclopamine (10μM) treatment decreased the number of cancer cells of about 50% (8505C, OCUT, CAL62) and 70-80% (TPC1, SW1736) whereas the same treatment did not have any effect on NTHY cells proliferation.

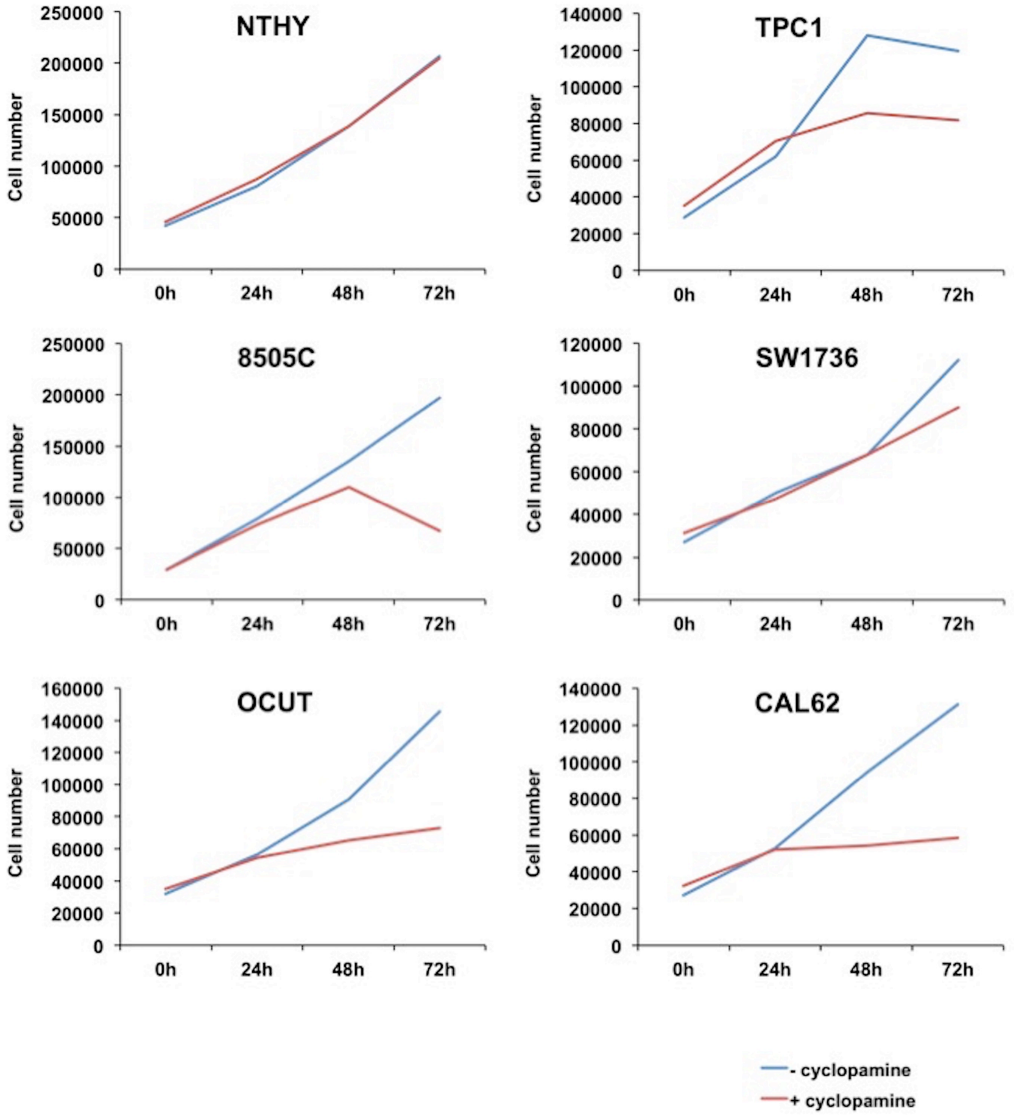


Figure 13. **Cyclopamine impairs thyroid cancer cells proliferation.** $3x10^4$ cells were plated and treated with cyclopamine 10μM. Cells were counted in triplicate every 24 hours. At 72 hours the number of thyroid cancer cells was reduced compared to untreated cells.

4.5 Silencing of Gli1 transcription factor in OCUT and CAL62 cells impairs cell migration

To characterize the function of Gli in thyroid cancer cells, we stably silenced Gli1 expression in OCUT and CAL62 cells by RNA interference (**Fig.14**). The efficacy of knockdown was proved by quantitative RT-PCR, showing that short hairpin RNA transfection (shGli1) reduced protein levels by about 70% in both cell lines (**Fig.14A**).

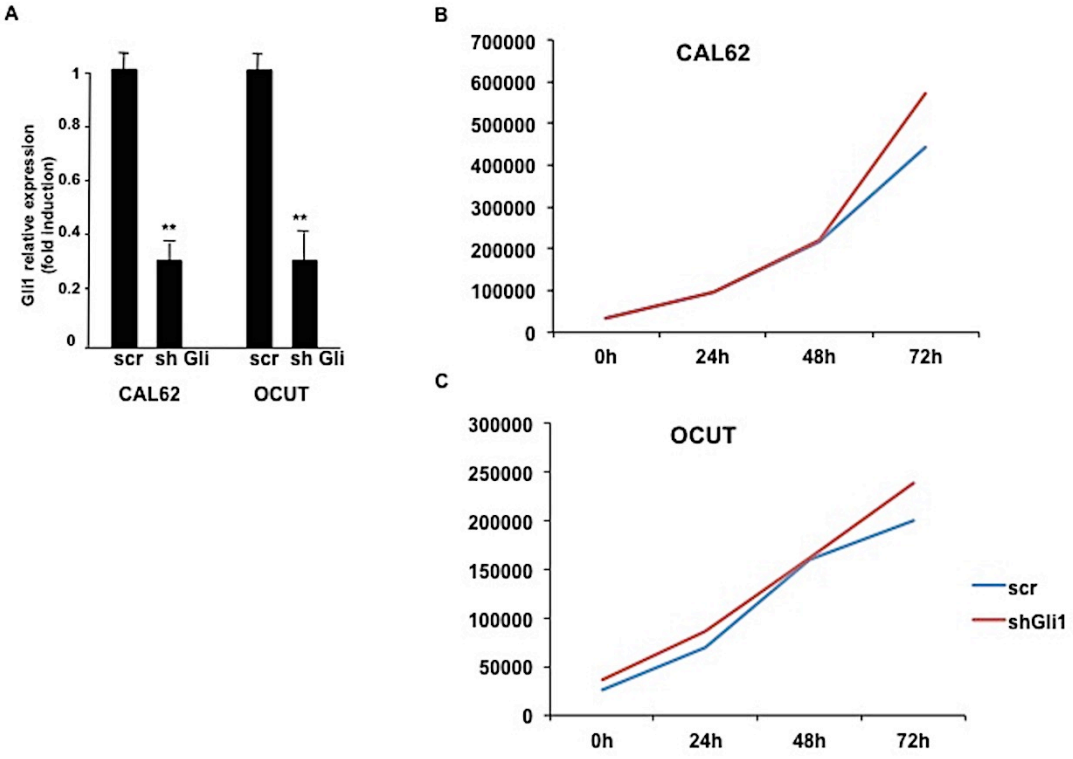


Figure 14. Gli1 silencing effect on cancer cells proliferation. A) CAL62 and OCUT cells were stably transfected with a short hairpin RNA (shGli1) or scrambled plasmid and mRNA expression levels were measured by Q-RT-PCR. Error bars represent standard deviations of experimental triplicates. **B)** CAL62 and OCUT cells stably silenced for Gli1 (shGli1) did not show reduction of proliferation compared to control cells transfected with scrambled sequence (scr).

Although we did not observe a clear reduction in cell proliferation, most likely for the activation of compensatory proliferative mechanisms in the stably silenced cells or for the presence of Gli2, that compensates the Gli1 silencing (**Fig.14B-C**), we discovered an impaired cell migration ability, by performing a wound healing assay. More specifically, a scraped wound was introduced on a confluent monolayer of OCUT and CAL62 stably transfected with shGli1 or with scrambled sequence and the cell migration into the wound was monitored

after 24 hours. As shown in Fig. 15, OCUT and CAL62 cells transfected with the scrambled sequence efficiently migrated into the wound while cells transfected with Gli1 shRNA showed reduced migration ability, suggesting a role for Shh signaling in regulating migration abilities of thyroid cancer cells.

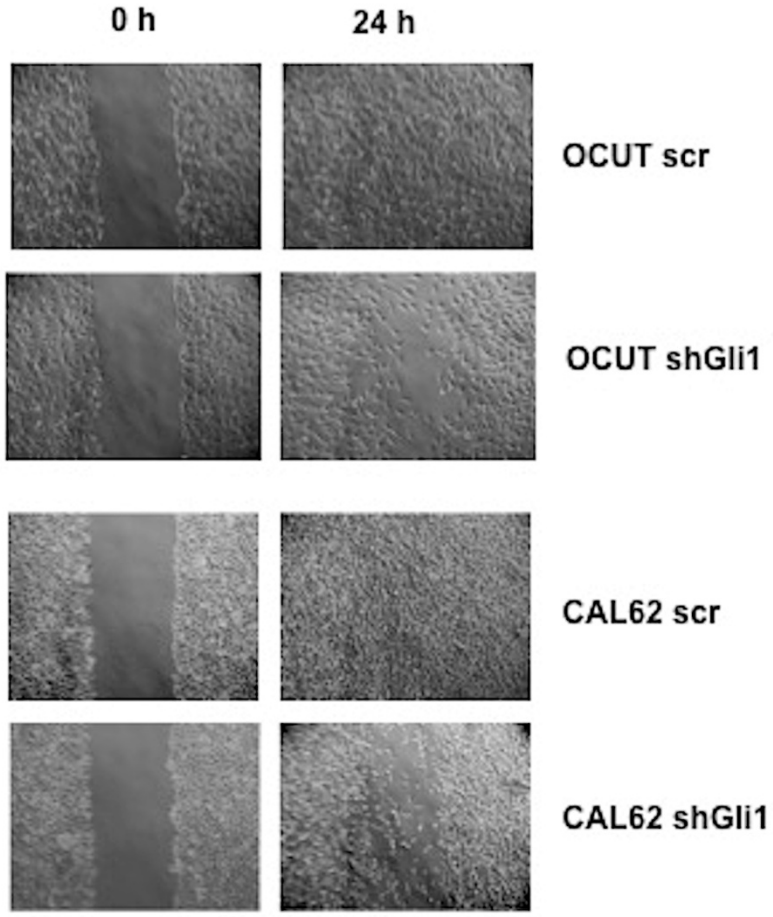


Figure 15. Silencing of Gli1 reduces the migration ability of OCUT and CAL62 cells. Cancer cells stably silenced for Gli1 were plated to form a confluent monolayer. A scratch wound was inflicted on cellular monolayer and after 24 hours cells were photographed. Silenced cells showed a reduction of migration ability.

4.6 Mechanisms of Sonic Hedgehog pathway activation in thyroid cancer cells

After demonstrating that the Shh pathway is expressed and active in thyroid cancer cells, we wondered what was the mechanism of activation. From the expression studies we noticed that in BCPAP cells the level of RNA expression of the Ptch1 receptor was particularly low (**Fig.6**); therefore we hypothesized a ligand independent activation in this cell line. To check whether

the low expression was due to gene deletion, we performed a gene dosage through a Copy Number Variation assay (CNV) for Ptch1in BCPAP cells and in control cell lines (**Fig.16**). Interestingly, when using NTHY cells as diploid control cells, we found a substantial gene dosage difference compared to BCPAP cells, suggesting a single copy deletion of Ptch1 gene.

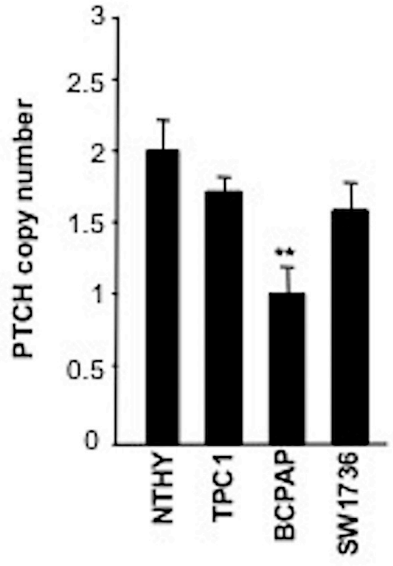


Figure 16. Ptch1 Copy Number assay. Ptch1 gene expression levels were analyzed by performing a Copy Number Variation assay (CNV) in a panel of thyroid cells. BCPAP cells showed a half reduced dosage of the Ptch1 gene compared to NTHY cells set as diploid control. Error bars represent standard deviations of experimental triplicates.

Because the RNA expression of Ptch was reduced by more than half in BCPAP cells (as shown in Fig.6), we asked whether there would be also gene methylation. To this aim, we treated BCPAP cells with the DNA methyltransferase inhibitor azacytidine (4 μM for 48 hours) (**Fig.17**). Interestingly, treatment with azacytidine recovered the Ptch1 expression suggesting gene methylation in BCPAP cells. All together these findings indicate that in BCPAP cells the Shh pathway could be activated in a ligand-independent way, through loss of Ptch receptor due to genetic deletion (on one allele) and gene hyper-methylation (on the other allele).

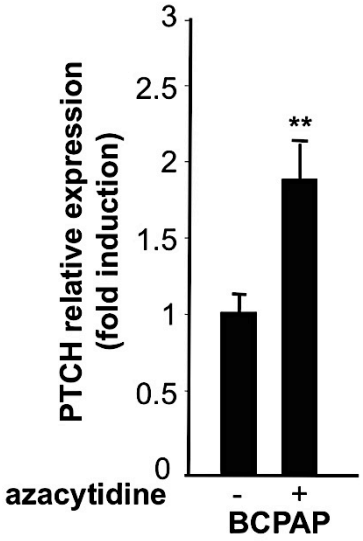


Figure 17. De-methylation treatment of Ptch1 gene in BCPAP cells. BCPAP cells were treated with azacytidine (4 μM) for 48 hours. After treatment Ptch1 mRNA expression levels were measured by Q-RT-PCR. Error bars represent standard deviations of experimental triplicates.

Because most of our cancer cell models express low levels of Shh ligand (**Fig.7**), we hypothesized a ligand independent activation of the pathway in these cells. Several evidences of a crosstalk between Shh signaling and other signal transduction pathways have been reported in different types of cancer (Rovida and Stecca 2015). As we know that our thyroid cancer cells are positive for RET or BRAF/RAS mutations, we decided to test whether these oncogenes would activate the pathway in our cell model, by performing Gli-Luc assay in embryonic kidney HEK293T cells transfected with activated forms of BRAF, RAS or RET/PTC1 plasmids (**Fig.18A**).

Our data, shown in figure 18A, suggest that all these oncogenes are inducing Gli-Luc activation. To confirm our findings, we also performed the Gli-Luc assay in a rat-derived differentiated thyroid follicular cell line (PC-Cl3) stably expressing PTC1 (RET/PTC1), BRAF (BRAFV600E) and HRAS (Gly12Val) oncogenes. As shown in figure 18B, transformed PC cells showed an increased activity of Gli-Luc reporter compared to wild type PC-Cl3 cells.

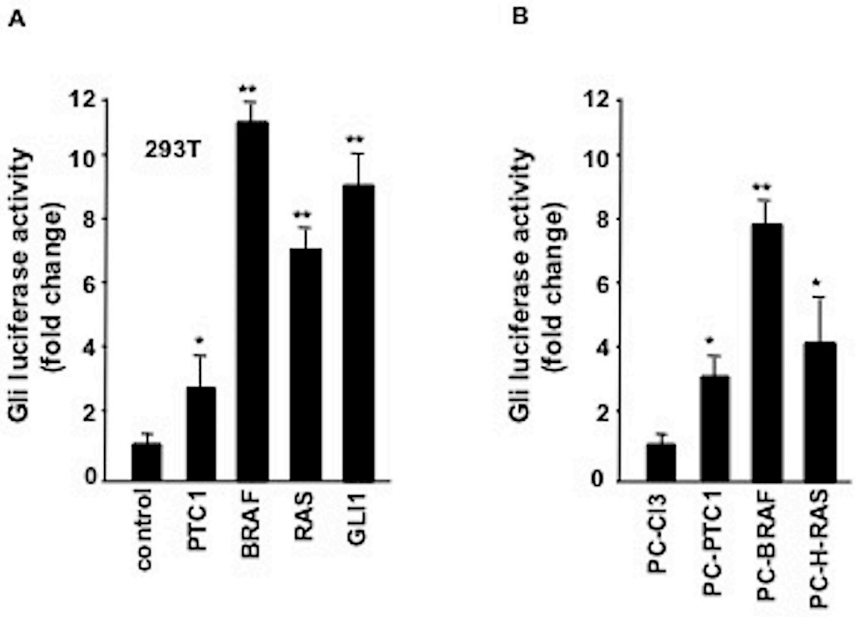


Figure 18. Oncogenic RAS/BRAF/MAPK and RET pathways activate Shh signaling. A) HEK293T cells were transfected with Gli-Luc reporter (pGL3-Gli-Luc) together with RET/PTC1 (PTC1), BRAFV600E (BRAF) and RASV12 (RAS) active forms and luciferase activity was measured 48 hours after transfection. Gli1 transfection was used as positive control. **B)** A similar experiment was performed also in rat PC-Cl3 cells stably expressing PTC1, BRAF and RAS active forms. Error bars represent standard deviations of experimental triplicates.

To check whether Gli-luc activation would result in increased Gli transcription, we performed Q-RT-PCR experiments in the same rat transformed cells. As shown in figure 19, PC BRAF cells at high levels and PC PTC1 cells at lower levels showed increased expression of Gli1 with respect to parental untransfected cells.

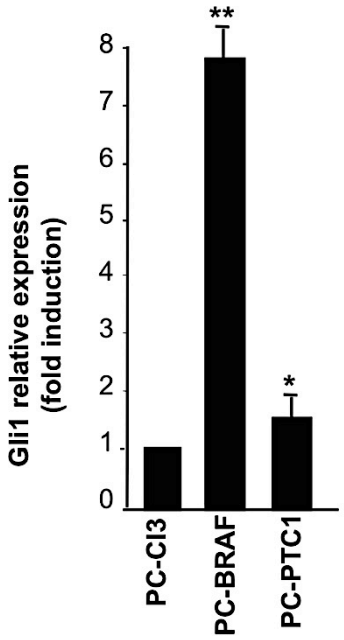


Figure 19. Gli1 is up-regulated in PC cells stably transfected with PTC and BRAF oncogenes. Gli1 mRNA expression levels were evaluated by Q-RT-PCR. PC-BRAF and PC-PTC1 showed high expression levels of Gli1 compared to PC control cells. Error bars represent standard deviations of experimental triplicates.

To confirm the interaction between Shh and BRAF or RET, we treated thyroid cancer cell lines with specific Protein Kinase Inhibitors (PKI) targeting these oncogenes (**Fig.20**). In particular, TPC1 (RET/PTC) positive cells were treated with Vandetanib (ZD-6474), a TKI that binds to the ATP-binding pocket of the RET kinase (at 500nM for 6 and 24 hours) (Knowles et al. 2006), while 8505C (BRAFV600E) cells were treated with Vemurafenib (PLX-4032), an ATP-competitive inhibitor of BRAF (at 500nM for 6 and 24 hours) (Bollag et al. 2010). As shown in figure 20, Gli-Luc was strongly reduced in TPC1 and 8505C cells treated with ZD-6474 and PLX-4032 respectively, in a time depending manner, suggesting interaction between RET and BRAF with Shh pathway.

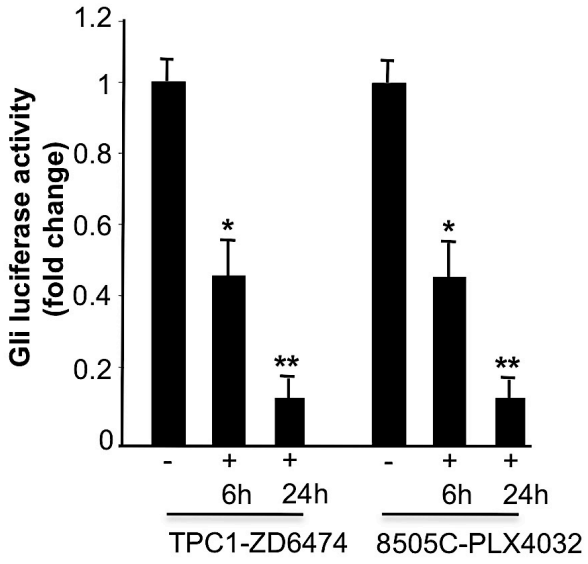


Figure 20. Inhibition of RET and BRAF oncogenic pathways impairs Gli-luc activity. TPC1 and 8505C cells were transfected with Gli-Luc reporter (pGL3-Gli-Luc). After 24 hours cells were treated with ZD-6474 and PLX-4032, respectively, at 500nM for 6 and 24 hours and Shh activation was evaluated by Luciferase assay. Inhibition of RET and BRAF pathways impaired Gli luc activation. Error bars represent standard deviations of experimental triplicates.

Similarly, Gli1 RNA expression levels were reduced in TPC1 and 8505C cells treated with ZD-6474 and PLX-4032 respectively, as shown in figure 20. All these findings suggest that oncogenic RET and RAS/BRAF/MAPK pathways interact with Shh pathway activation in thyroid cancer cells, generating a ligand independent (but partially Smo-dependent), non canonical mechanism of activation.

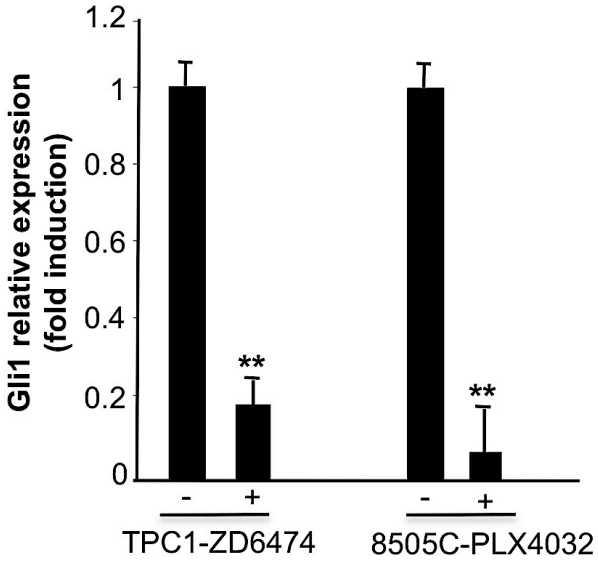


Figure 21. Gli1 expression levels are supported by RET and RAS oncogenic pathways. TPC1 and 8505C cells were treated with ZD-6474 and PLX-4032, respectively, at 500nM for 24 hours. After drug treatment, mRNA expression levels were evaluated by Q-RT-PCR. Treated cells showed a strong reduction of Gli1 expression levels. Error bars represent standard deviations of experimental triplicates.

4.7 Sonic Hedgehog pathway activation in tumor-stroma interaction

Because it has been demonstrated, in different cancer models, that Sonic Hedgehog is one of the main signaling pathway activated in stroma and involved in cancer-stroma interaction, and because in our cells we did not observe high expression of the Shh ligand, although the pathway is active and responsive to ligand (**Fig.9**), we decided to investigate the role of Shh pathway in regulating the interaction between thyroid cancer cells, in particular anaplastic cells, and tumor fibroblasts. Interestingly, when checking the Shh ligand expression levels in IMR90 fibroblasts we discovered an higher production of Shh, compared to anaplastic CAL62 and OCUT cells (**Fig.22**).

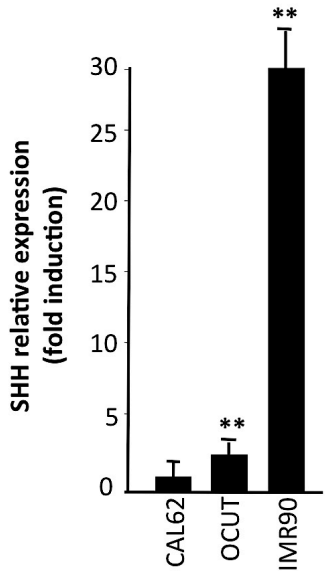


Figure 22. Shh ligand is highly expressed in IMR90 fibroblasts. Shh mRNA expression levels were evaluated by Q-RT-PCR. IMR90 cells showed high expression levels of Shh ligand compared to CAL62 and OCUT control cells. Error bars represent standard deviations of experimental triplicates.

In order to study if the Shh produced from fibroblasts could activate Shh pathway in cancer cells, we stimulated OCUT and CAL62 cells with the conditioned medium derived from IMR90 cells as well as with the conditioned medium of three different human primary cultures of thyroid mesenchymal stromal cells (MSC) (Hematti 2012; Keating 2012) and measured the expression levels of several components of the Shh pathway. As shown in figure 23A, both thyroid cancer cell populations, upon stimulation, showed increased protein levels of Smo receptor (that most likely in not inhibited by Ptch and therefore not internalized for degradation) with respect to unstimulated (control) cells. Moreover, stimulated CAL62 showed increased amount of Gli1 nuclear protein level (checked by cell fractionation) while in OCUT cells we observed the up-regulation of nuclear Gli2 transcription factor with respect to control cells (**Fig.23B**).

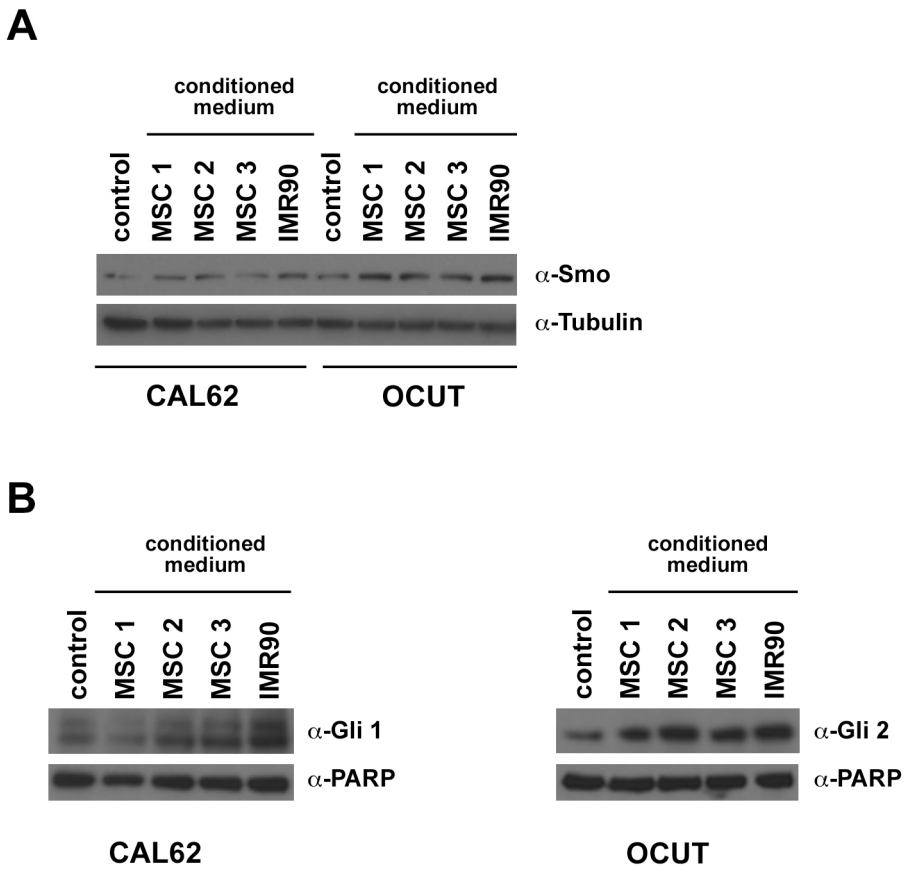


Figure 23. Stromal cells stimulation induces up-regulation of Shh signaling components in anaplastic thyroid cancer cells. CAL62 and OCUT cells were stimulated with conditioned medium derived from IMR90 and mesenchymal cells for 24 hours. After stimulation total cell lysates (50μg) were analyzed with anti-Smo, and total tubulin level was used for normalization. Nuclear cell lysates (30μg) were analyzed with anti-Gli1 and anti-Gli2. Total Parp level was used for normalization.

To then test whether the increased Gli levels were paralleled by an increased transcriptional activity, we performed a Gli-Luc assay in CAL62 cells upon MSCs or IMR90 co-cultures. For this experiment, stromal cells orIMR90 fibroblasts were plated on culture dishes and Gli-Luc transfected cancer cells were plated on cover glasses co-cultured in the same dish and then used for luciferase reading. Figure 24 shows that stroma co-culture increases Gli transcriptional activity in CAL62 cancer cells.

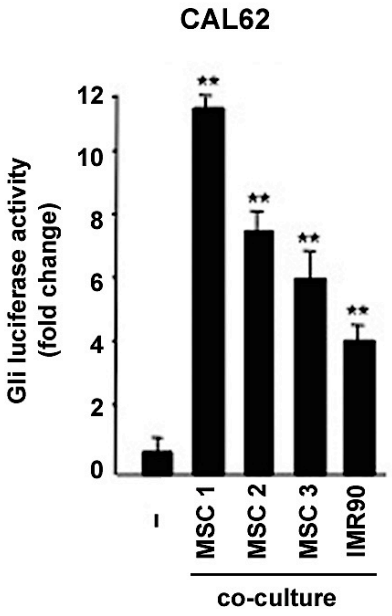


Figure 24. Stromal cells stimulation induces Shh signaling activation in anaplastic thyroid cancer cells. $3x10^4$ Cal62 cells were plated on cover glass and transfected with Gli-Luc reporter (pGL3-Gli-Luc). After 24 hours cells cover glasses were put in co-culture with mesenchymal cells or IMR90 fibroblasts for 24 hours. Shh pathway activation was evaluated by Luciferase assay. Stromal stimulation increases Shh signaling activation compared to unstimulated cells. Error bars represent standard deviations of experimental triplicates.

All together, these results suggest that the Shh ligand secreted by stromal fibroblasts is able to activate in paracrine manner the Shh pathway in thyroid cancer cells.

4.8 Sonic Hedgehog pathway activation from stroma cells supports thyroid cancer cells invasion, migration and non-adherent growth

To study the effect of stromal fibroblast stimulation on the biological properties of thyroid cancer cells, we first tested cell proliferation by performing growth curve analysis of cancer cells stimulated or not stimulated with conditioned medium derived from fibroblasts and did not observe significant differences (data not shown). We therefore investigated the ability of stimulated cancer cells to invade Matrigel. To this aim, stromal cells (IMR90 and MSCs) were plated into the bottom of a transwell dish while OCUT and CAL62 cells were seeded into the top chamber of transwells coated with Matrigel. As shown in Fig. 25 (upper panels) the presence of stromal cells allowed cancer cells to invade the Matrigel. To address the role of the Shh pathway in this stimulation, we performed the same experiment in the presence of cyclopamine (10 μM). Fig. 25 (lower panels) demonstrates that the inhibition of Smo (with cyclopamine) reduced the ability of stromal cells to attract cancer cells to invade

the matrigel, indicating the involvement of Shh pathway in cancer-stroma interaction.

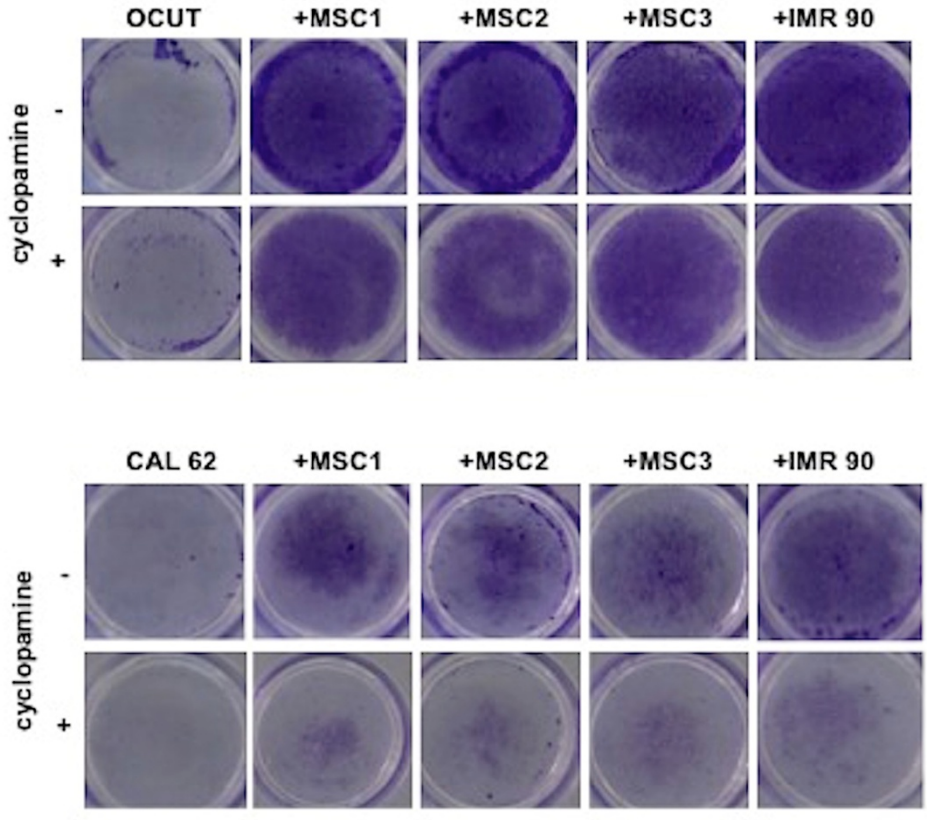
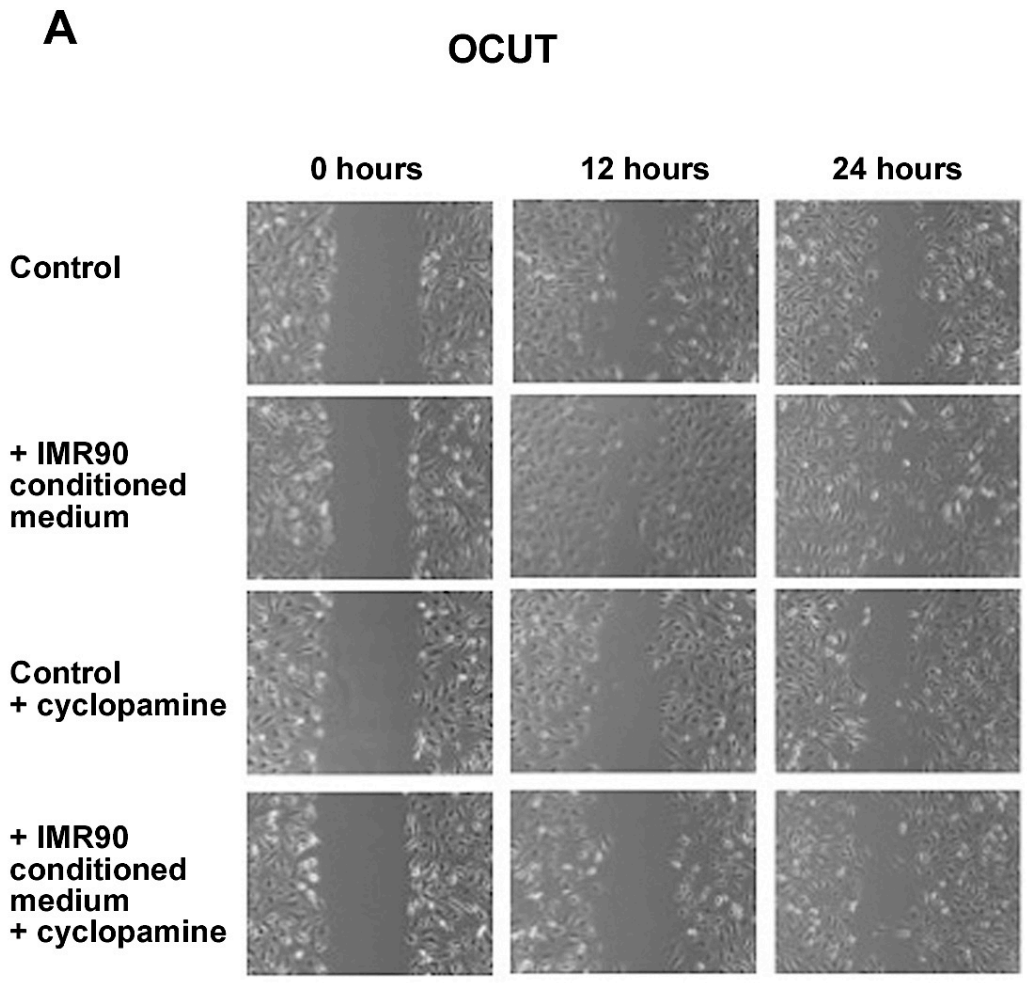


Figure 25. Shh pathway controls the ability of stromal cells to stimulate Matrigel invasion of ATC cells. $2X10^5$ Stromal cells were plated on the bottom of wells while OCUT or CAL62 cells ($2X10^5$) were plated into the upper chamber. The lower chamber was filled with complete medium. Cyclopamine (10μM) was added to cancer cells (upper chamber) and to stromal cells (bottom of well). After 48 hours cells were fixed, stained and photographed. Cancer cells, in the presence of stromal cells, showed increased invasion ability compared to control cells. Cyclopamine treatment impaired the effect of stromal cells on cancer cells invasion abilities.

To study also the migration properties of stimulated cancer cells, we performed a wound healing assay by measuring the ability of CAL62 and OCUT cells to close an artificial wound. Our results, in Fig 26A and B, show that cancer cells stimulated with stroma conditioned medium have increased cell migration ability with respect to control non stimulated cells, because the cells are able to close the wound at earlier time points. Again, cyclopamine treatment (10 μM) impaired this effect of stroma on cancer cells.

A


OCUT
0 hours
12 hours
24 hours
Control
+ IMR90 conditioned medium
Control + cyclopamine
+ IMR90 conditioned medium + cyclopamine

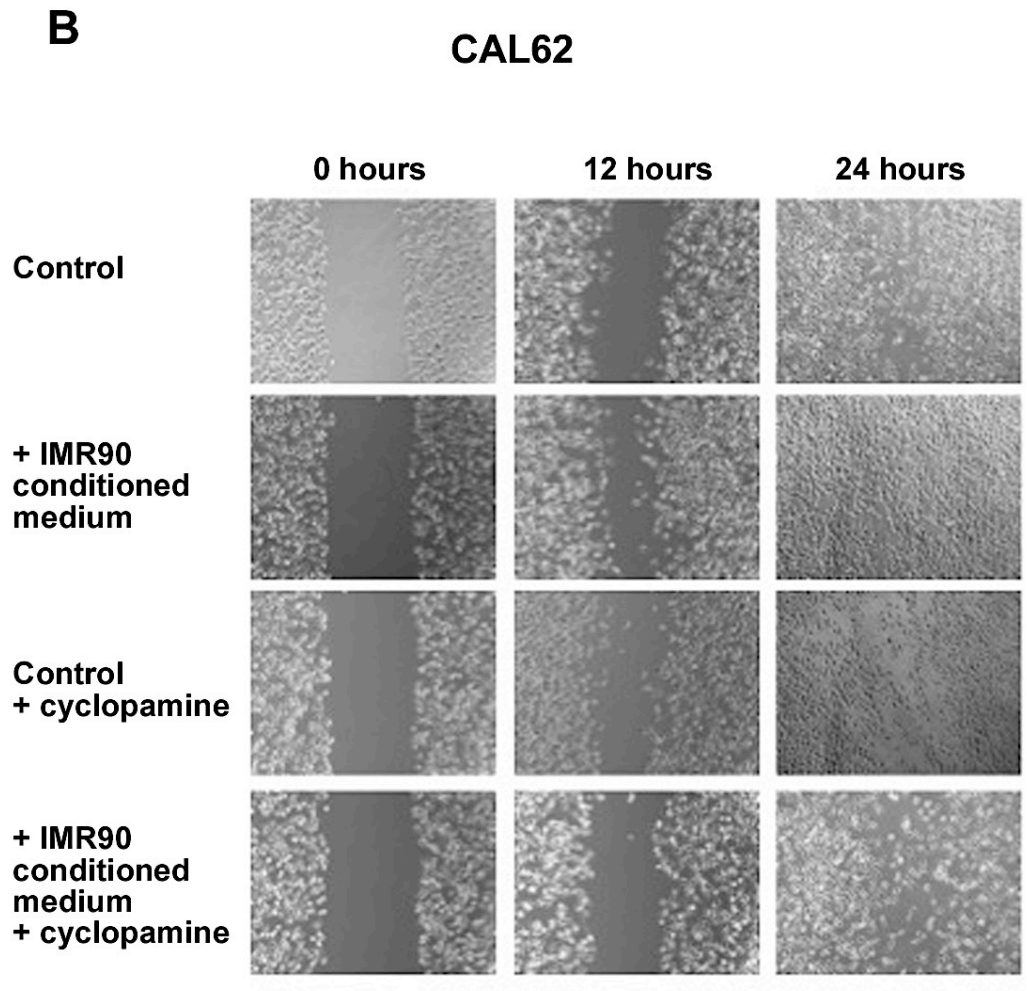


Figure 26. Stromal cells stimulation increases cancer cells migration ability. Cancer cells were plated to form a confluent monolayer. A scratch wound was inflicted on cellular monolayer. Cancer cells were stimulated with conditioned medium derived from IMR90. Cells were photographed after different times. Stimulated cells showed a an increased migration ability compared to unstimulated cells. Cyclopamine treatment (10μM) reduced cancer cells migration compared to control cells.

Finally, to recreate the 3-dimensional organization of tumor-stroma interaction, we cultured CAL62 cells alone or together with stromal cells (IMR90 and MSC) under non-adherent and low serum conditions. As shown in figure 27, IMR90 and MSCs alone did not assemble into aggregates (data not shown) while CAL62 cancer cells were able to grow in suspension and form spheroid-like complexes. Interestingly, co-culture of thyroid cancer cells with IMR90 or MSC cells showed the formation of larger spheroids while cyclopamine treatment (10 μM) had a dramatic effect on reducing the size of these cell aggregates (**Fig. 27).**

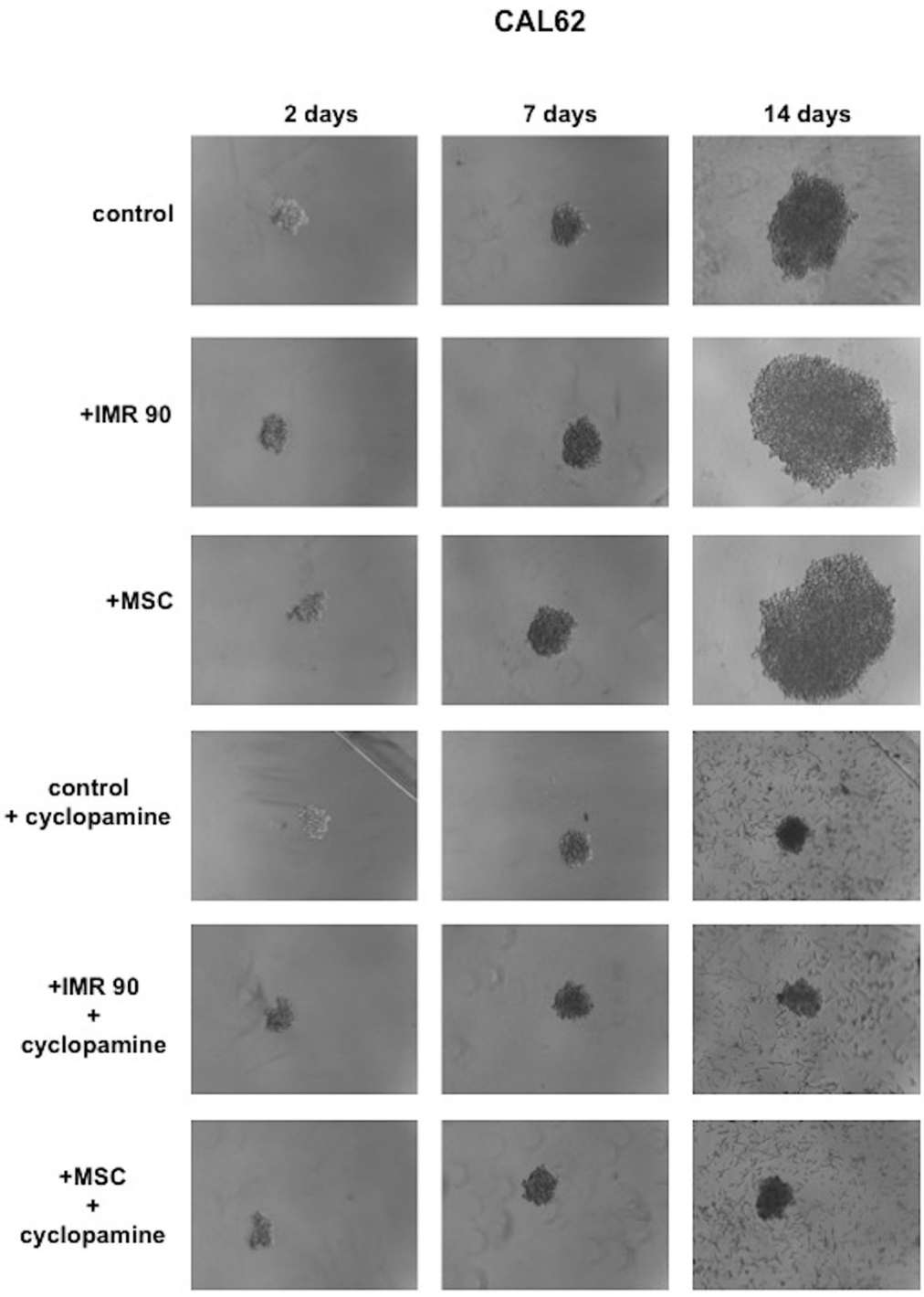


Figure 27. Stromal cells support cancer cells growth in non adherent conditions. CAL62 cells (50 cells/well) were 62 on ultra-low-attachment 96-well in low serum medium alone or together with MSC or IMR90 cells (20 cells/wells). Spheroids were photographed after 7 and 14 days. Cancer cells, supported by stromal cells, formed larger spheroids and this ability was impaired by cyclopamine treatment (10μM).

All together, these data suggest that tumor stromal fibroblasts, through Shh pathway activation, support thyroid cancer cells invasion, migration and ability to grow in non adherent/low serum conditions.

To finally test whether the co-culture with cancer cells had some influence on the stromal counterpart, we stimulated IMR90 fibroblasts with the supernatant derived from OCUT and CAL62 cells and tested the amount of Shh production. Interestingly, upon cancer cells stimulation, IMR90 showed an increased level of Shh RNA (**Fig.28A**), suggesting that cancer cells can boost the Shh production from stromal cells. The produced ligand could either act in a paracrine way on cancer cells as well as it could act in an autocrine way on stromal cells themselves, because our data demonstrate that IMR90 cells have a functional Shh pathway as they respond to a recombinant Shh (pSecShh) and to the stimulation with CAL62 and OCUT supernatant (**Fig.28 B, C**).

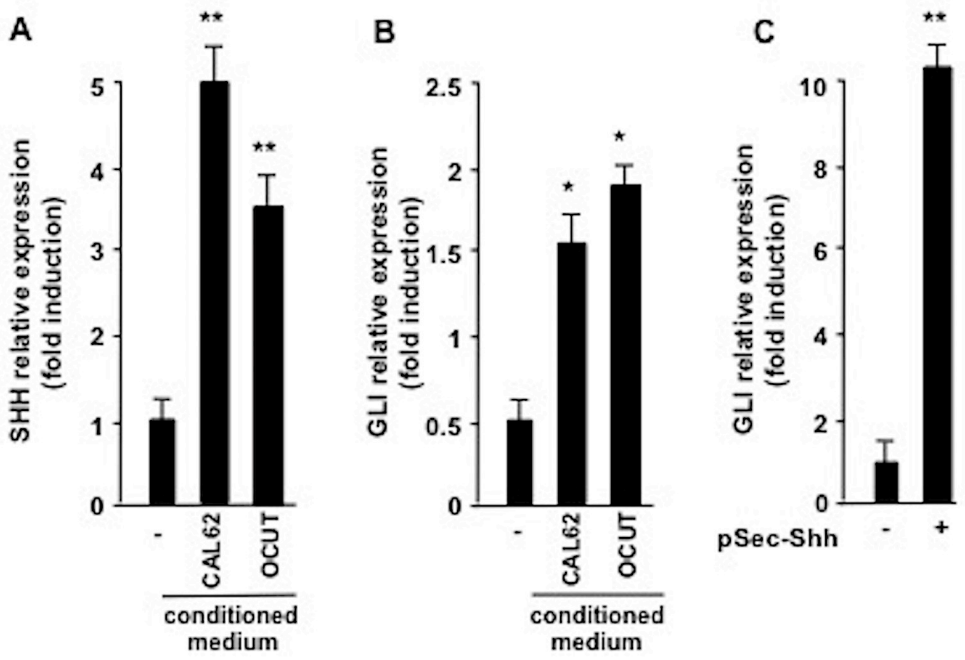


Figure 28. Effect of CAL62 and OCUT cells on IMR90 fibroblasts. A) Q-RT-PCR of Shh in IMR90 upon stimulation with conditioned medium derived from CAL62 and OCUT cells. Q-RT-PCR measuring the expression of Gli1 in IMR90 stimulated with cancer cells conditioned medium **(B)** or with Shh ligand **(C).**

5.0 DISCUSSION

The Hedgehog pathway is a key regulator of embryonic development, tissue maintenance and repair, although recent evidences have highlighted its involvement in several forms of cancer (Hahn et al. 1996; Amakye et al.2013). Different mechanisms of Shh pathway activation have been proposed in cancer (Ingham and McMahon 2001). Ligand independent Shh pathway activation has been described in some familial cancers, such as basal cell carcinoma, medulloblastoma and rhabdomyosarcoma, as a consequence of genetic aberrations, targeting mainly the Ptch inhibitory receptor (Pandolfi and Stecca 2015). Ligand independent non canonical Shh pathway activation has also been reported in other tumor models as a result of a crosstalk between Shh signaling and different tumorigenic pathways (Rovida and Stecca 2015). Ligand-dependent autocrine or paracrine activation, has been involved in tumor progression and maintenance of other cancers, including gastrointestinal, lung, breast and prostate cancers. The paracrine mechanism can either be due to Shh secretion by cancer cells and promotion of Gli transcriptional activity in stromal cells (direct paracrine), or to Shh ligand secretion by stroma activating the pathway in cancer cells (reverse paracrine mechanism) (Bailey et al. 2009). Many evidences suggest in fact that the Sonic Hedgehog is one of the main pathways involved in tumor-stroma interaction. This finding is interesting because tumor microenvironment and tumor-stroma interaction have recently been highlighted as new frontiers in studying the cancer biology as well as in discovering novel therapeutic approaches to target tumors.

In this Dissertation, we have addressed the role of the Sonic Hedgehog pathway in thyroid cancer and its involvement in thyroid tumor-stroma interaction. Several components of the Shh pathway have been demonstrated by others (Dong et al. 2014; Nelson et al. 2010) and by ourselves to be up-regulated in thyroid tumors and cell lines (Fig. 5, 10, 11). Here we have shown that in thyroid cancer cell lines the Gli transcriptional activity is higher compared to control cells and that cyclopamine treatment impairs this activation (Fig. 8, 9). To then understand the mechanisms regulating Shh signaling in other cell models, we have performed several experiments suggesting the existence of a ligand independent activation, due to Ptch loss, in BCPAP cells (Fig. 16, 17) and a non canonical activation, through the cross-talk between Shh signaling and other oncogenic pathways (such as RAS/BRAF/MAPK and RET pathways) in our cells lines, positive for these oncogenes (Fig. 18-21). We have also demonstrated that in thyroid cancer cells the Shh pathway can be activated through a ligand dependent paracrine mechanism, with Shh ligand produced by stromal cells and inducing Gli activation in cancer cells (recerse paracrine). Our co-culture experiments and stimulation with conditioned-medium suggest that stromal cells (IMR90 and MSCs) support cancer cells invasion, migration and non-adherent growth abilities in a Shh dependent manner (as cyclopamine impairs these effects) (Fig. 23-27). Interestingly, we also observed an increased

Shh production in fibroblasts stimulated with conditioned medium derived from cancer cells, suggesting a bi-directional paracrine effect (Fig. 28).

Our evidences suggest therefore that the Hedgehog pathway can be considered as a novel prognostic marker and therapeutic target to be used alone or in combinatorial protocols in aggressive thyroid cancers. Combinatorial strategies are increasingly expanding in the treatment of several types of cancer characterized by non-canonical Shh signaling activation. For instance, a combined inhibition therapy of Shh pathway and MEK or AKT, has shown to produce synergistic effects in reducing melanoma and cholangiocarcinoma cell proliferation *in vitro* (Stecca et al. 2007; Jinawath et al. 2007). Moreover, Smo inhibitors, in combinatorial strategies with EGFR inhibitors, have been described in several preclinical models. In pancreatic cancer cells for instance, EGFR inhibitor gefinitib in combination with cyclopamine, has shown a tumor growth decrease and apoptotic rate increase (Chitkara et al. 2012) while in prostate cancer cells, cyclopamine in combination with gefitinib and docetaxel treatment has shown an inhibitory effect on proliferation and invasiveness (Mimeault et al. 2007). Stromal cells are able to support tumorigenesis and, at the same time, protect cancer from the effect of chemotherapeutic drugs. Therefore, combination treatments targeting both the stroma and the cancer cells with Shh inhibitor IPI-926, together with chemotherapy drug gemcitabine, has shown depletion of stroma and increased drug delivery in a preclinical mouse model of pancreatic cancer proving efficacy of combinatorial strategy against both cancer cells and stroma (Olive et al. 2009). At the moment there are no clinical studies in thyroid models demonstrating the correlation between stroma inhibition and reduced aggressiveness. We are confident that our data can shed new light on the possibility to target Shh pathway in thyroid cancer cells as well as in tumor microenvironment, suggesting novel therapeutic possibilities to fight aggressive anaplastic cancer for which currently there is no effective therapy.

6.0 CONCLUSIONS

In conclusion, here we show that Shh pathway is involved in thyroid neoplastic transformation supporting cancer cell proliferation, migration and invasion abilities. We hypothesize the existence of ligand independent mechanisms: in BCPAP cells that do not express the Ptch receptor and do not respond to Shh (1) as well as in other cell lines positive for RAS/BRAF or RET activating mutations where cross-talk mechanisms with Shh signaling, have been decribed to interefere with PKA inhibitory mechanisms and increase Gli amount as well as nuclear localization and transcriptional activity, promoting thyroid cancer cell growth (2) (Rovida and Stecca, 2015). Moreover, we demonstrate the presence of ligand dependent inverse paracrine activation mechanisms, where Shh ligand is secreted by stromal cells and acts in a paracrine way on cancer cells to support invasion, migration and tumorigenicity (3) (**Fig.27**).

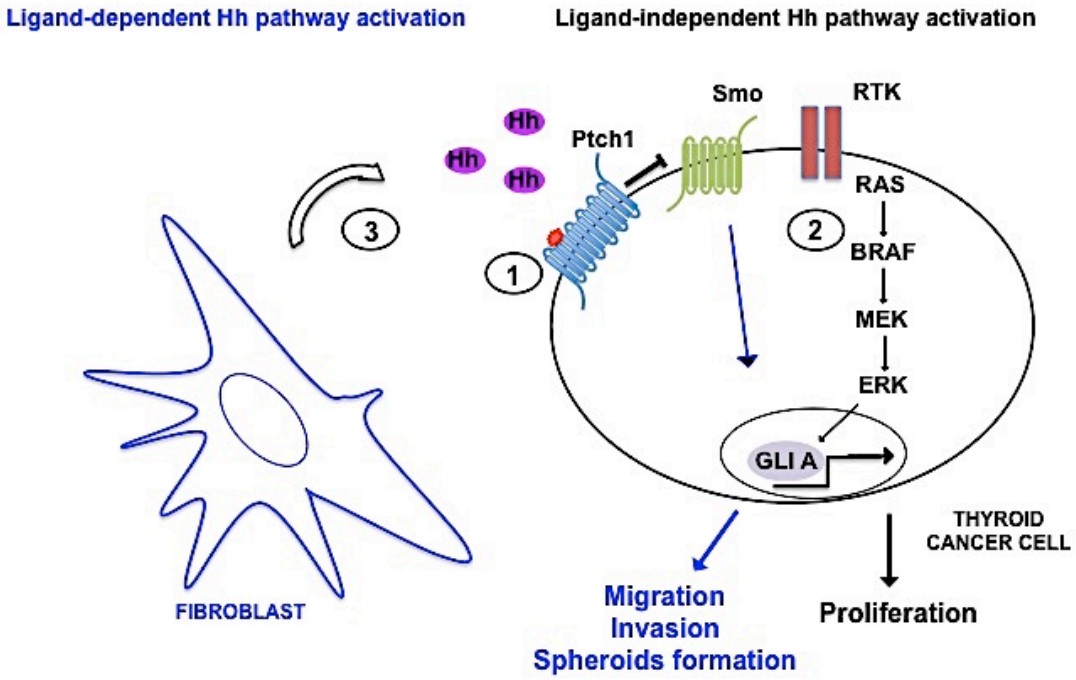


Figure 29. Schematic representation of Shh pathway activation mechanisms in thyroid cancer cells.

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LIST OF PUBLICATIONS

1. A Parascandolo, M Santoro, MD Castellone – Sonic hedgehog in thyroid tumors- *in preparation* (main body of this dissertation)

2. MC Cantisani, A Parascandalo, M Perälä, C Allocca, V Fey, N Sahlberg, F Merolla, F Basolo, MO Laukkanen, OP Kallioniemi, M Santoro, MD Castellone – A Loss-of-Function Genetic Screening Identifies Novel Mediators of Thyroid Cancer Cell Viability , Oncotarget, *in press*

In this study we have performed a loss of function screening by transfecting thyroid cancer cells (TPC1 cells) with a library of siRNAi targeting the whole human kinome in order to discover kinases involved in thyroid cancer cell viability. Our data identify a novel set of thyroid cancer regulators, includeing several members of EPH receptor tyrosine kinase family as well as SRC and MAPK (mitogen activated protein kinases) families.

3. G Vecchio, A Parascandolo, C Allocca, A Strazzulli, M Moracci, C Ugolini, F Basolo, MD Castellone, M Santoro, N Tsuchida - Downregulation of FUCA-1 in human thyroid anaplastic carcinomas - *in preparation*

In this work we have studied the role of the alpha-L-Fucosidase-1 (FUCA-1) in thyroid cancer. This enzyme is involved in the removal of fucose fom glycans and has been shown to be downregulated in highly aggressive and metastatic histotypes of human cancers. Our data demonstrate that the expression is significantly reduced in anaplastic thyroid cancer (ATC) as well as thyroid cancer cell lines. Moreover, the transfection of FUCA-1 in ATC cells is able to impair their tumorigenic properties.