TSH Signalling and Cancer

revisão

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ABSTRACT

Thyroid cancers are the most frequent endocrine neoplasms and mutations in the thyrotropin receptor (TSHR) are unusually frequent. Here we present the state-of-the-art concerning the role of TSHR in thyroid cancer and discuss it in light of the cancer stem cell theory or the classical view. We briefly review the gene and protein structure updating the cancer related TSHR mutations database. Intriguingly, hyperfunctioning TSHR mutants characterise differentiated cancers in contrast to undifferentiated thyroid cancers which very often bear silenced TSHR. It remains unclear whether TSHR alterations in thyroid cancers play a role in the onset or they appear as a consequence of genetic instability during evolution, but the presence of functional TSHR is exploited in therapy. We outline the signalling network build up in the thyrocyte between TSHR/PKA and other proliferative pathways such as Wnt, PI3K and MAPK. This network's integrity surely plays a role in the onset/evolution of thyroid cancer and needs further research. Lastly, future investigation of epigenetic events occurring at the TSHR and other loci may give better clues for molecular based therapy of undifferentiated thyroid carcinomas. Targeted demethylating agents, histone deacetylase inhibitors combined with retinoids and specific RNAis may help treatment in the future. **(Arq Bras Endocrinol Metab 2007;51/5:654-671)**

Keywords: Thyrotropin; Cancer; Signalling; MAPK; PI3K; PKA; Wnt; Thyroid; NIS

RESUMO

Sinalização de TSH e Câncer.
Os cânceres de tiróide são as neoplasias endócrinas mais frequentes e as mutações no receptor de tirotrofina (TSHR) são incomumente frequentes. Nesta revisão nós apresentamos o "estado da arte" com relação ao papel do TSHR no câncer de tiróide e o discutimos à luz da teoria da célula matriz do câncer ou a visão clássica. Revisamos brevemente a estrutura do gene e da proteína, atualizando a base de dados das mutações do TSHR relacionadas ao câncer. Curiosamente, mutações do TSHR com hiperfunção caracterizam cânceres diferenciados, em contraste com os cânceres de tiróide indiferenciados, os quais muito comumente mostram TSHR silenciados. Permanece obscuro se as alterações do TSHR em cânceres de tiróide têm algum papel no surgimento ou se elas aparecem como conseqüência da instabilidade genética durante seu desenvolvimento, mas a presença de TSHR funcional é explorada na terapia. Nós delineamos a rede de sinalizacão desenvolvida no tirócito entre TSHR/PKA e outras vias proliferativas como a Wnt, PI3k e MAPK. A integridade desta rede certamente tem um papel no surgimento/evolução do câncer de tiróide e necessita de novas pesquisas. Finalmente, novas investigacões sobre os eventos epigenéticos que ocorrem no TSHR e outros locais poderão trazer novas informações para uma terapia de base molecular nos carcinomas indiferenciados de tiróide. Agentes demetilantes direcionados, inibidores da histona-deacetilase, combinados com retinóides e RNAs específicos poderão auxiliar no tratamento futuro. **(Arq Bras Endocrinol Metab 2007;51/5:654-671)**

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THYROID CANCER: THE FREQUENCY, THE TYPES AND THE HYPOTHESIS

THYROID CANCER IS THE MOST frequent endocrine neoplasia and represents 1% of all cancers. According to the American Cancer Society, the frequency of thyroid cancer in USA was 100 per million population in 2003, this incidence has been increasing at more than 5%/yr for a decade mostly due to increased diagnosis of small tumours (1). The annual mortality from thyroid cancer in 2003 was 5–6 per million. The discrepancy between incidence and mortality reflects the good prognosis for most thyroid cancers. Thyroid neoplasms may appear as benign nodules and adenomas or malignant tumours that can be from follicular cell origin: differentiated or undifferentiated; from the parafollicular C cells: medullary thyroid carcinoma (MTC), or else. Differentiated carcinomas are hystologically divided into papillary thyroid carcinoma (PTC) or follicular thyroid carcinoma (FTC). Detailed descriptions of this classification can be consulted elsewhere, i.e.: http://www.meb.uni-bonn.de/cancer.gov/CDR0000062913.html. Most thyroid tumours are sporadic as a consequence of somatic mutations, although hereditary thyroid carcinoma resulting from germinal mutations also occurs. Currently there are 2 hypotheses to explain thyroid cancer onset that are summarised in figure 1. The classical view, depicted on the right, considers thyroid carcinoma as a complication of a pre-existing follicular adenoma accumulating mutations, which drive the progression through a de-differentiation process. Differentiated thyrocytes

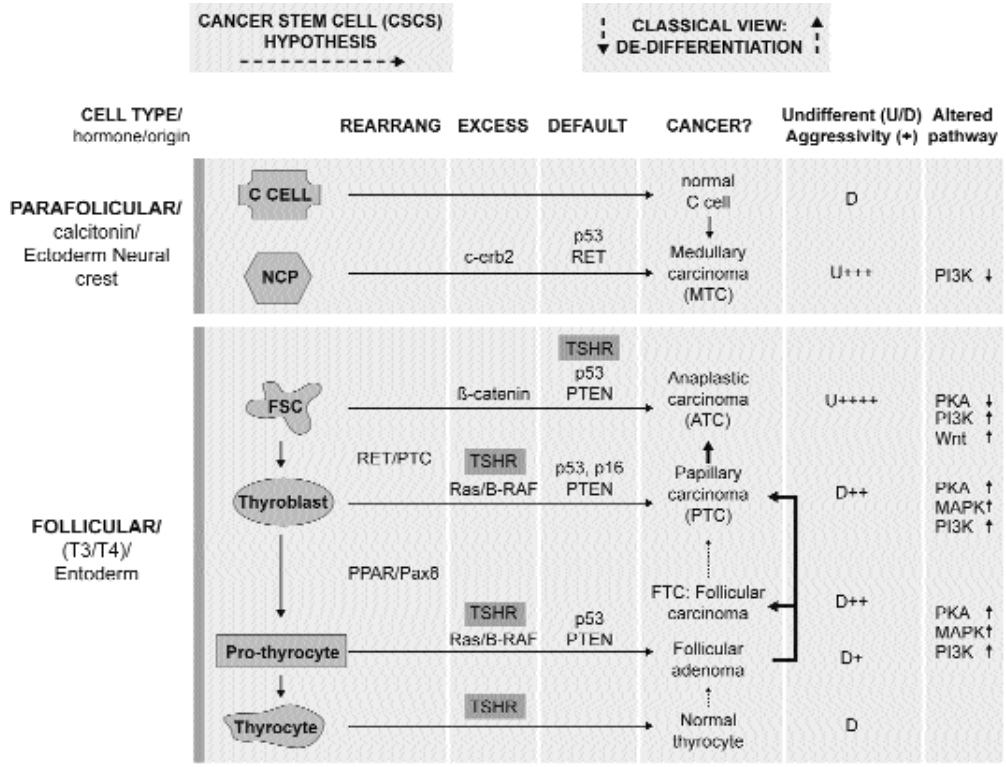

Figure 1. Two hypotheses to explain the onset of thyroid cancers: The Cancer Stem Cell hypothesis is represented on the left side: thyroid cell precursors may encounter mutations that deviates them from the normal differentiation path (vertical arrows) to develop into cancer stem cells (horizontal arrows). Thyroid cell precursors are abbreviated as NCP (Neural Crest Precursor) or FSP (Follicular Cells Precursor) to stress their different origin and characteristics. NCP migrate long distances to populate the thyroid and perhaps retain migrating (invasion) characteristics that enable them to become malignant with a smaller number of mutations. Mutations leading from every precursor to a different type of cancer are depicted on the horizontal arrows and are classified in (i) large chromosomal rearrangements, (ii) gain of function or excessive levels and (iii) silencing or disabling mutations in the proteins described. These mutations would lead from the precursor of a thyroid cell to a cancer cell (either latent or proliferative). The classical hypothesis is represented to the right of the horizontal arrows. This view assumes a process of de-differentiation represented by vertical arrows leading from differentiated thyrocyte at the bottom to the most undifferentiated: the Anaplastic Thyroid Cancer. The mutations described before would correspond now to the vertical arrows leading from one type of cancer to another. The column previous to the last represent the degree of differentiation (D) or undifferentiation (U) together with the aggressiveness of the cancer, which will be proportional to the number of "+" symbols. The last column resumes the signalling pathways represented by the stated protein mutants and whether the path is enhanced (↑) or interrupted (↓).

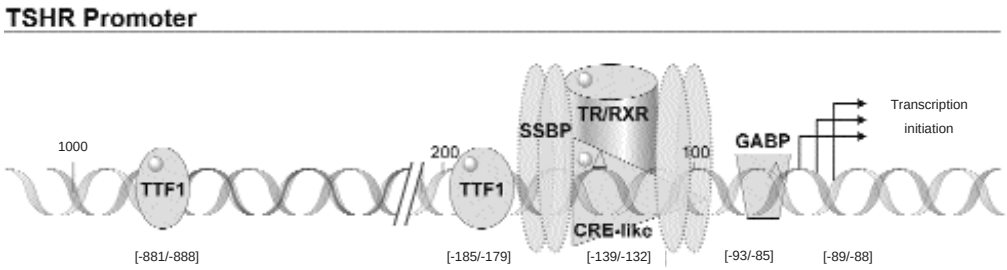

Figure 2. Known regulatory elements in the promoter of the TSHR: The promoter of TSHR is TATA-less and has multiple transcription start sites (arrows). GA-binding protein (GABP) dimers binding are sensitive to DNA methylation. The cAMP-response element (CRE-like) encompasses a constitutive enhancer that may bind the activators CREB or ATF2 and the repressor ICER. Binding of single strand binding protein (SSBP to the 5′ and 3′ flanking decanucleotide repeats modulates CRE-like site activity. The CRE-like site overlaps with a thyroid hormone response element. Binding of the heterodimer TR/RXR (thyroid hormone receptor and retinoid X receptor) to this site represses transcription. The proximal promoter bears a binding site for the thyroid transcription factor 1, TTF1 and there is at least another binding site at the far 5′ end. The positions in the promoter relative to the transcription start site appear depicted in brackets underneath the sites. ● Inside of a transcription factor indicates modulation via phosphorylation; Δ indicates modulation via methylation.

develop into differentiated thyroid cancer first, then undifferentiated and eventually to anaplastic thyroid carcinoma (ATC). Early thyroid tumour development correlates with mutation of signalling molecules encoded by five alternative genes: Ras, Ret, trk, gsp, and the TSH receptor; additional mutations in a genome caretaker gene such as p53 ensure genomic instability and would drive evolution towards ATC. Discrete de-differentiation steps difficult to explain are required and also it is rather infrequent that a benign adenoma evolves towards carcinoma; at present, it seems that most thyroid carcinomas are malignant from the onset. The original Ras and Ret mutations seen in FTC and PTC are hardly seen in ATC. These and other observations, particularly from Chernobyl irradiation studies, led to the formulation of an alternative hypothesis based in the existence of cancer stem cells (CSCs) and represented by the horizontal arrows from left to right in figure 1. The CSCs would be derived from embryonic thyroid stem cells, thyroblasts or pro-thyrocytes intermediaries in the pathway of differentiation accumulating mutations that lead to carcinogenesis. The CSCs hypothesis proposes that thyroid cancer develops as a consequence of a block in a differentiation step from any of these intermediaries rather than the process of de-differentiation contemplated by the classical view. The phenotype and incidence of a thyroid cancer type would remember those of the originating CSCs i.e.: the most undifferentiated and aggressive ATC would develop from the earliest and less abundant thyroblasts and would have the lower rate of incidence (2-4). The establishment of ES cell cultures able to differentiate into thyrocytes may clarify these views (5).

THYROTROPIN BINDING TO ITS RECEPTOR IS THE MAIN STIMULUS FOR THYROCYTE PROLIFERATION

Thyrotropin (TSH) secreted by the pituitary and bound to its receptor (TSHR) regulates thyroid growth and differentiation at late developmental stages but is not responsible for organogenesis or cell migration according to the phenotypes of the TSHR knockout mice [(6,7), reviewed in (8,9)]. TSH promotes growth of the thyrocyte directly binding to its receptor and also indirectly, stimulating secretion of autocrine growth factors and amyloid precursors (10-12) or the expression of growth factor receptors or of vascular endothelial growth factor, VEGF (13). Additional growth factors, such as insulin/IGF-1 or serum factors (14,15) may be required for TSH induced proliferation of the thyrocyte. Thus, paracrine and autocrine factors secreted by follicular cells, the stromal apparatus and lymphocytes may be implicated in initiation and perpetuation of thyroid hyperplasia. TSH and its receptor are required not only for proliferation in the thyrocyte but also for the expression of differentiation markers such as thyroglobulin, thyroperoxidase or the Na/I symporter (NIS) that is responsible for iodide uptake. These differentiation markers are required for the correct function of thyrocytes that is the synthesis of thyroid hormones. TSHR molecules in the membrane are quite stable and signalling in the thyrocyte will be controlled mainly through circulating TSH levels. TSH secretion is inhibited via negative feedback by thyroid hormones; in the absence of thyroid hormones there will be hyper-secretion of TSH and abnormal thyrocyte proliferation.

THE RELEVANCE OF TSHR FOR THE ONSET/ EVOLUTION OF THYROID CANCER IS UNCLEAR

The importance of the TSHR signalling for the onset/evolution of thyroid cancer is supported by experiments in which regained expression of functional TSHR in a follicular thyroid cancer cell line (HTC) reduced angiogenesis and size of tumours of xenotransplanted HTC cells (16). Hyperactivated TSHR is commonly found in most adenomas, less common in differentiated carcinomas and the gene is silenced in undifferentiated cancers such as ATC (table 1, see also figure 1). From the point of view of the classical de-differentiation hypotheses, these data from case studies are difficult to interpret; two mutation events on the TSHR gene are highly improbable, but would be required in the evolution from adenoma to ATC: first hyperactivating mutations in adenomas and later silencing in ATC. It would be easier to imagine that the TSHR gene being a susceptible candidate for mutation would mutate differently in different precursor cells undergoing transformation to CSCs. According to the CSCs hypothesis, only one alteration would be required at the TSHR locus to render each transformed phenotype. TSHR implication in the onset of thyroid cancer remains unclear but its functionality is important for thyroid function and treatment of thyroid cancers (see below). Additionally, TSHR may also be relevant in diagnosis. For example, based on the fact that most thyroid cancers still express the TSHR (17), its mRNA has been used as a highly sensitive and specific marker to detect thyroid cancer cells in peripheral blood (18). Differentiated thyroid cancer has better prognosis because the cells express NIS and can be targeted by radioactive ^{131}I. TSH administration to thyroid cancer patients with functional TSHR ensures NIS expression, uptake of ^{131}I and the removal of malignant cells left over after ablation of the cancerous gland or nodule. Moreover, differentiated cancers produce thyroid hormones maintaining reduced pituitary TSH secretion. Undifferentiated thyroid carcinomas expressing TSHR but not NIS exist demonstrating that TSHR is required but not sufficient for NIS expression. These cancers are rather difficult to target because radioactive ^{131}I will not be taken and insufficient thyroid hormone production will result in elevated TSH levels inducing further proliferation even in cells with diminished TSHR numbers.

Hence, signalling from the TSHR may play a role in the onset, evolution, diagnostic and therapies of thyroid cancer.

THE TSHR GENE AND PROTEIN

The TSHR cloned in 1989 (19-22) belongs to the family of the G protein coupled receptors (GPCR) and

Table 1. Update of "gain of function" mutations encountered at the TSHR in thyroid cancer and hyperthyroidism.

AA-codon	LOCATION	PHENOTYPE	Comments	REF
S281N/T/G	Ectodomain	In vitro studies	involved in G protein interaction	(131)
G431S	TM1	Hyperthyroidism	increased cAMP (Gas) and I3P(Gaq)	(132, 133)
V463M	TM2	hyperthyroidism		(134)
I486F	ECL1	adenoma/FTC	ECL1is involved in silencing unstimulated TSHR	(123, 135)
I486M	ECL1	Follicular adenoma		(135)
S505R/N	TM3 (GL)	Hyperthyroidism	Interactions TM3- TM5 are important for	(114, 136, 137)
V509A	TM3 (GL)	Congenital Hyperthyroidism, Adenoma, follicular cancer	silencing the unstimulated receptor. Increased cAMP (Gas) and I3P(Gaq).	(126)
L512R	TM3	PTC		(138)
L512Q	TM3	Hyperthyroidism Multinodular goiter	gain of function	(139) ; (140)
V556F	TM4	HTN	Increased cAMP (Gas) and I3P(Gaq)	(141)
I568V	ECL2	In vitro studies; non autoinmune hyperthyroidism		(45, 142)
V597F	TM5	Thytoxicosis and hyperthyroidism		(143)
Δ613-621	IC3	Toxic thyroid nodules	critical for G protein interaction	(144)
T620I	IC3	FTC		(145)
A623I	IC3 (S+GL)	increased cell proliferation TSH independent		(146)
M626I	TM6 (S+GL)	Non autoimmune Hyperthyroidism	increased basal cAMP production	(44)
I630M	TM6 (6)	Hyperfunctioning thyroid nodules		(147)
F631V	TM6 (S+GL)	Hyperfunctioning thyroid nodules	Constitutive activation of Ga/cAMP	(148)
F631S	TM6	non autoinmune hyperthyroidism		(149)
F631I	TM6	Toxic FTC		(150)
D633Y	TM6	Toxic FTC		

contains 7 transmembrane (TM) domains anchored to the basolateral plasma membrane of the thyrocytes and a number of other cells. The TSHR promoter contains functional binding sites for several transcription factors including GABP (23), TTF1 (24), TR/RXR (25), CREB and ICER (26), nevertheless there is little fluctuation in the TSHR mRNA levels and regulation of functional TSHR is mainly exerted at the postranslational level (27), specially by glycosylation and correct folding at the ER (28).

The mature TSHR is encoded by a single gene with 10 exons (29,30). The protein contains 2 subunits: a large ectodomain also called A or α subunit is encoded by exons 1–8 and binds TSH; a short transmembrane and intracellular domain encoded by exons 9–10 called B or β subunit that will interact with G proteins to initiate signalling. Postranslational intramolecular cleavage of a 50 aa chain in the ectodomain close to the membrane and reduction of disulphide bridges releases the A and B subunits (29) followed by shedding of the A subunit from the membrane bound receptor (31,32). The presence of the TSHR ectodomain inhibits an otherwise constitutively active β subunit (33) and the interactions between the ectodomain (α), the extracellular loops and transmembrane domain TM6 (in the β subunit) are critical for the maintenance of an inactive state (34). Figure 3 recapitulates the main features in the structure of TSHR. The white dotted line overlapping TM5 and TM6 marks the position of the most frequently found mutations in adenomas. The ectodomain consists mainly of 9 leucine rich repeats (LRR) with interconnecting loops important for receptor structure and activation (35). Precise delineation of the TSH binding pocket of the receptor has been made through deletion-mutation analysis and a panel of antibodies (36,37). The β subunit contains the 7 TM domains joined by extracellular loops (ECL) and intracellular loops (ICL), and interacts selectively with G proteins when the TSHR is activated (34,38). Unstimulated TSHRs form oligomers that return to the monomer state with TSH (39,40). TSHR oligomerisation is an early posttranslational event detectable by FRET (fluorescent resonance energy transfer) in the ER-Golgi (41). After TSH binding, a constitutively oligomeric TSHR dissociates into active monomers, which will be subsequently recruited to the lipid rafts to interact with G proteins (42,43). Systematic mutagenesis of the β-subunit has been used to identify critical residues and mechanisms of interactions as illustrated by the following examples. A repulsive separation between TM6 and TM3 in the context of a constitutively active TSHR mutant indicated the opening of the cytoplasmic face of TSHR for G protein coupling (44); mutations in ECL2 and TM6 residues revealed dynamic interactions able to increase or decrease basal TSHR activity (45); deletions and substitutions at the N-terminus of ICL2 served to study TSHR coupling to

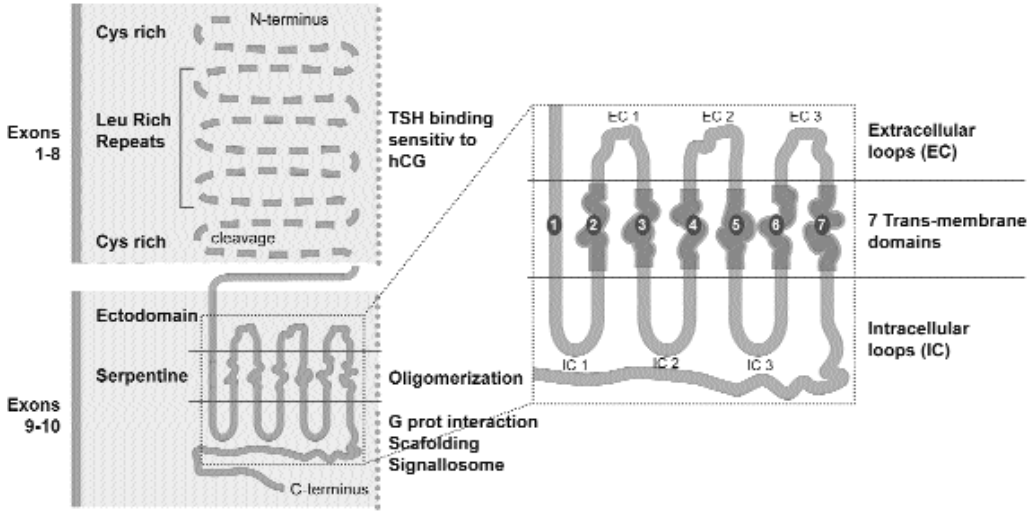

Figure 3. Structure of the TSHR protein: The ectodomain (N-terminus) appears outside the membrane and the serpentine domain through the membrane. On the left the exons coding different regions are denoted. The dashed folded line represents the molecule shedded after the cleavage. Structural domains are highlighted: Cys rich regions and Leucine Rich Repeats. To the right of this scheme the functional significance of each domain is ascribed, (hCG) denotes Corionic Gonadotropin because TSHR may also bind it.The serpentine (C-terminus) is amplified at the right highlighting the 7 transmembrane domains denoted as a number in a circle, the intra cellular loops (ICLs), important for G protein coupling, and the extracellular loops (ECLs), important for basal and activated function. White dots encompassing the 5th–6th transmembrane domains and the 3rd ECL represent the area of the TSHR where most of the activating mutations related to thyroid cancer are described.

both G_α and G_q and to demonstrate that ICL2–ICL3 interactions are critical for selective G_q activation (34).

Upon TSH binding, cytoplasmic proteins such as β-arrestins bind and desensitize GPCRs uncoupling them from G proteins and promoting their internalization. TSHR binds β-arrestin 2 but does not colocalize with β-arrestins in endosomes (46). β-arrestins effects are pleiotropic and have been shown to act also in signal transduction and in transcription. β-arrestins scaffold some activated GPCRs with the Raf/MAPK/ERK cascade inhibiting nuclear translocation of ERK (47) and some β-arrestins translocate to the nucleus and associate with transcription cofactors such as p300 and CBP at the promoters of genes targeted by the transcription factor CREB (cAMP-Response Element Binding Protein) (48). After desensitizing, TSHR is rapidly internalised by clathrin-coated pits (49,50). Most endocytosed receptors recycle back to the membrane, a vital process to maintain the levels. An adaptor protein, hScrib, is crucial to maintain the correct number of TSHR molecules and to scaffold a correct signalling complex. hScrib interacts with TSHR at the cytoplasmic end of the basolateral membrane of the thyrocyte inhibiting basal TSHR internalization. Upon internalisation, hScrib interaction with the internalised TSHR promotes its recycling. To fulfil this function, hScrib interacts with the C-terminus of the TSHR and recruits several enzymatic activities including GTPase activating proteins and Guanine nucleotide exchange factor (GEF) in a complex required for receptor recycling (51). Finally we must mention here a phenomenon termed "specificity crossover" that consist in the binding and activation of TSHR not only by TSH but also by closely related hormones such as luteinizing (LH) and corionic gonadotropin (hCG) (52,53), a phenomenon that may become important in pathologies that curse with excessive hCG secretion. Indeed variants of hCG with increased TSHR affinity have been described in some patients (54).

THE CLASSICAL PATHWAY AND THE NETWORK THAT FRAMES TSH ACTION IN THE THYROCYTE

Thyrocyte growth occurs mainly through the TSHR mediated increase in cAMP. cAMP-dependent protein kinase (PKA) activation will follow as described below:

1. TSH stimulated TSHR dissociates the heterotrimeric G protein activating the $G_{\alpha s}$ subunit.

2. $G_{\alpha s}$-dependent activation of adenilyl cyclase (AC) follows, increasing cAMP production.

3. cAMP-dependent activates PKA by dissociation of its regulatory subunits.

4. Activated PKA phosphorylates target proteins including membrane receptors, signalling molecules and transcription factors changing their activities to promote growth and differentiation. The variety of targets will further amplify and diversify the final outcome of this pathway. Perhaps the most classical target for PKA after translocation of its catalytic subunit to the nucleus is the transcription factor CREB, whose transcriptional activity will be promoted upon phosphorylation by PKA.

Every intermediary in the pathway described may additionally interact with side molecules belonging to other pathways. This will build up a network responsible for refining every response according to a contextual environment. Altered wiring of this network interconnecting TSHR/PKA with other proliferation pathways such as PI3K, BRaf/MAPK or Wnt plays a pivotal role in cancer, and common targets such as CREB or cyclin D1 may need the integrity of the network to be accurately regulated. We will describe next the classical pathway outlining possible important side branches for thyroid cancer, but since it is out of the scope of this review to analyse them in detail, excellent reviews on each pathway can be consulted elsewhere. Figure 4 outlines the main effectors of TSHR and interactions with other signalling pathways.

STEP 1: The TSHR stimulated by TSH interacts with heterotrimeric G proteins

TSH induced dissociation of heterotrimeric G proteins leads to G_α and $G_{\beta\gamma}$ activation. There are many subtypes of α and βγ subunits, each combination activating a different set of pathways, and there are excellent reviews on the subject [i.e.: (55)].

G_α proteins

Photoaffinity labelling of the TSHR followed by inmunoprecipitation suggests that TSHR interacts with all four G_α subtypes ($G_{\alpha s\ (L)}$, $G_{\alpha s\ (S)}$, $G_{\alpha q}$, $G_{\alpha 11}$, $G_{i1\text{-}3}$, G_0 and G_{12}) (56), however TSHR signalling in the thyrocyte is mainly mediated by $G_{\alpha s}$ and $G_{\alpha q}$ coupling to increased cAMP production and phosphoinositide turnover respectively. $G_{\alpha s}$ stimulation of AC will increase cAMP, whose major effector is PKA. This path is further analysed below and activating mutations of the TSHR and $G_{\alpha s}$ that increase AC activity have been identified in hyperfunctioning benign follicular adenomas and less commonly in hypofunctioning adenomas and carcinomas of the thyroid. $G_{\alpha q/11}$ coupling to TSHR in rat FRTL-5 and human thyrocytes

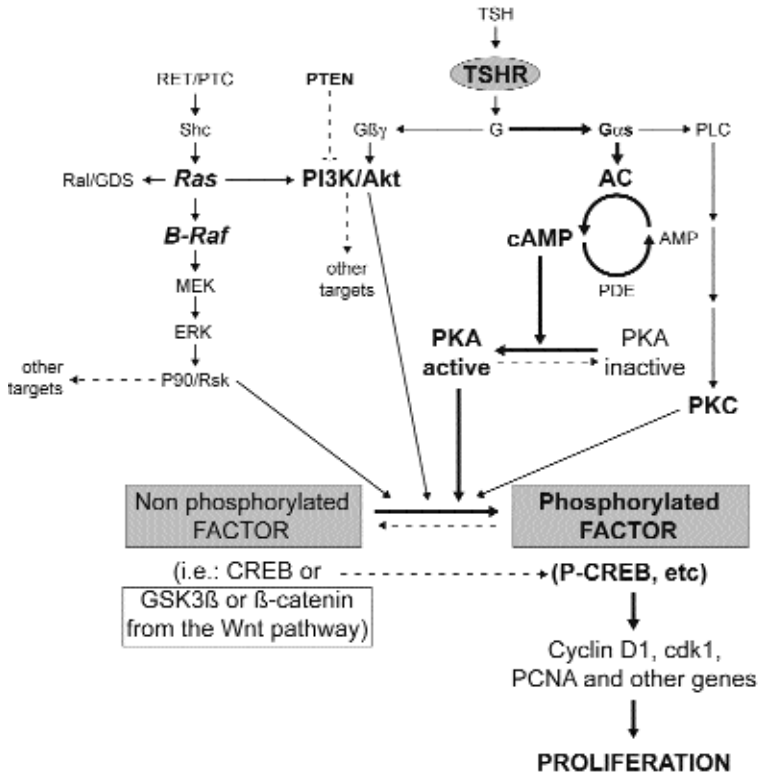

Figure 4. Classical TSHR signalling pathway and the framing network in thyrocyte proliferation. The bold arrows represent the classical TSHR signalling pathway towards proliferation. Normal arrows integrate crosstalking molecules from other signalling pathways. Dashed lines represent other targets that may or may not be related to this pathway. Commonly altered molecules in thyroid cancer that may alter the integrity of the signalling network are enclosed in a square. Examples of integration between the classical TSHR/PKA and the MAPK/ERK, PI3K/Akt and Wnt/β-catenin pathways are provided.

stimulates phospholipase C (PLCβ) (57,58), which catalyses hydrolysis of phosphatidil inositols in the membrane yielding di-acyl-glycerol (DAG) and inositol tri phosphate (IP3) as second messengers. DAG directly stimulates PKC. IP3 increase cytosolic Ca^{+2} levels (59), which act through a number of effectors including PKC. PKC stimulation is important in thyroid cancer because it is the major effector of tumor promoters such as phorbol esters, and its activation leads to proliferation and de-differentiation in FRTL-5 and PC CL3 thyrocytes (60,61). TSHR mediated activation of the PLC-Ca^{+2} cascade has been controversial because it requires very high TSH concentrations in human primary thyrocytes and in FRTL-5 cells (62-64), however, TSHR clearly increases Ca^{+2} mobilizations at least in certain contexts such as repeated stimulation or simultaneous activation of other GPCRs (65). Increases in PLC-PKC activities have been reported in thyroid carcinomas (66) but it could be due to activation of other receptors because TSHR is poorly expressed in neoplastic tissue (67); moreover, there is a negative feed back from PKC to PLCβ in thyroid carcinomas (68), and constitutively active mutants of $G_{\alpha q}$ have never been found in thyroid neoplasms (69), although the mutation induces thyroid hyperplasia in mice. Hence, the significance of the TSHR-$G_{\alpha q}$-PLC-PKC in thyroid cancer still remains obscure. Different G proteins compete for binding of the TSHR (70) mainly $G_{\alpha s}$ or $G_{\alpha q}$ in thyrocytes which activate the PKA or PKC pathways respectively. Functional interference between the cAMP/PKA and PKC pathways has been described in normal thyrocytes (71,72) and in hyperfunctioning thyroid adenoma bearing $G_{\alpha s}$ mutants that induce the cAMP cascade and suppress the PLC-Ca^{2+} signalling (73). Transgenic mice expressing G_{α} mutants that constitutively activate both AC and PLC suggest that these cascades may cooperate in vivo towards development of thyroid follicular malignancies (74).

Finally, TSHR may also couple to $G\alpha_{i1\text{-}3}$, which will inhibit AC and decrease cAMP levels providing a mechanism to desensitise the TSHR.

$G_{\beta\gamma}$ activation

GPCR activated $G_{\beta\gamma}$ dimers may induce the MAPK and PI3K signalling pathways, which are involved in processes important for tumorigenesis such as proliferation and cytoskeletal remodelling. The diversity of $G_{\beta\gamma}$ subunits would allow at least 60 possible combinations, but the composition of the $G_{\beta\gamma}$ dimers in thyrocytes has not been examined in that detail. More than 20 effectors have been reported for $G_{\beta\gamma}$ activation including phospholipases (75), ACs (76), other GPCRs and PI3Ks (77). In the thyrocyte, $G_{\beta\gamma}$ directly activates PLCβ and PKC, which activates the PKB/Akt pathway. Our lab has shown that TSHR mediated $G_{\beta\gamma}$ activation directly stimulates PI3K in rat thyrocytes leading to diminished NIS expression (Zaballos, MA et al. 2007 submitted).

In summary, TSHR coupling to G proteins provides the opportunity for activation of the PKA, PKC and PI3K pathways, but mutations in G proteins hardly correlate with thyroid cancers, and hot thyroid nodules with constitutively active TSHR mutants very often bear reduced G_{α} protein expression presumably as a mechanism of desensitizing the TSHR (78).

STEP 2: TSHR-dependent activation of $G_{\alpha s}$ stimulates the AC increasing cAMP levels

cAMP has cell type specific effects and the outcome on proliferation is largely attributed to cross talk with the Ras-Raf-MAPK-ERK pathway. Besides PKA, a number of cAMP effectors may cross talk with the MAPK pathway in the thyrocyte mainly GTP-exchange factors (GEFs or Epac). Finally, other cAMP effectors in the thyrocyte include cAMP-gated membrane ion channels (79), and some phosphodiesterases (PDEs) such as PDE4. Steady state cAMP levels result from production by AC and degradation by PDEs. cAMP-PKA-dependent PDE4 activation feeds back negatively providing a mechanism to stop the signal (80). Given the enormous variety of cAMP effectors, our understanding of this very complex pathway is far from complete.

cAMP-dependent PKA activation by binding to its regulatory subunits is analysed in "Step 3" and here we briefly discuss other cAMP effectors. Although PKA activation in thyroid cells is necessary for cAMP mitogenic effects (15), it is not sufficient (81). In fact, cAMP inhibits growth in some human thyroid tumoral cell lines (82,83) perhaps involving negative feed back mechanisms such as over expression of PDE4, as described for autonomous hyperfunctioning thyroid nodules bearing constitutive activation of the cAMP pathway (84). In autonomous hyperplasic thyroid adenomas with constitutive activation of the cAMP pathway by TSHR and $G_{\alpha s}$ mutants is not sufficient to generate toxic thyroid adenomas (85). Cooperation with other signalling pathways initiated by insulin/IGF-1, bFGF, EGF or serum factors (14,15) is required by TSH in thyrocytes to display full mitogenic activity. Other cAMP effectors include GEF or Epac, which activate the small GTPases Rap1, Rap2 and Ras, providing another mechanism for diversification and tuning of the signal. The thyrocyte is highly enriched in Epacs (86-88). GTP bound Rap1 may activate Raf-1, B-Raf or C-Raf leading to activation of MAPK/*ERK1/2* or *p38 MAPK* and mitogenesis; Rap1 is over expressed in thyroid follicular cancer (89), and mutations in its effector B-Raf are present in most PTC. Activated Ras signalling may lead to *MAPK* or to *PI3K/PDK1* activation, both pathways being involved in mitogenesis. Ras is required for cAMP dependent mitogenesis in several rat thyroid cell lines such as FRTL-5 and WRT (90,91) and in 60–70% follicular thyroid adenomas, and carcinomas components of the RET/Ras/B-RAF signalling pathway are mutated (92-96). The role of this pathway is further discussed under the epigraph "Networking the TSHR" and has been widely reviewed elsewhere.

STEP 3: cAMP-dependent activation of PKA

PKA is composed of 2 catalytic and 2 regulatory subunits that bind cAMP. To date at least 3 different catalytic and 4 regulatory subunits have been found and depending on the combination the corresponding PKA molecules will be targeted to cytosolic substrates (PKA I) or to the membrane of certain organelles (PKA II) via a family of proteins called A kinase anchoring proteins or AKAPs (97). Selective activation of the cytosolic or membrane anchored forms of PKA in the thyrocyte has demonstrated specialised functions. PKA I stimulation increased iodide uptake in FRTL5 cells without affecting gene transcription while selective PKA II activation induced gene transcription and proliferation in FRTL5 cells (98).

Mutations that activate the catalytic subunits or inactivate the regulatory subunits would lead to the constitutive PKA activity found in endocrine tumors but so far no mutants for the catalytic subunits have been correlated to endocrine tumors. Inactivating mutations in the regulatory subunit RI leading to PKA I stimulation has been related to benign endocrine neoplasms, functioning nodules and follicular carcinomas of patients with Carney complex (99). However, selective PKA I activation by cAMP analogs showed anti proliferative activity in BRAF-mutated cells. PKA

II appears to be more clearly involved: silencing its regulatory subunit RIIβ impairs TSH nuclear effects, and loss of RIIβ expression in three human cancer cell lines suggests an essential role for PKA II in TSHR-mediated proliferation (98).

The transcription factor CREB is the classical nuclear target of PKA, although it is also targeted by other pathways, and PKA also targets other nuclear factors as we will see below.

STEP 4: PKA dependent activation of the transcription factor CREB

The ubiquitous transcription factor CREB binds to cAMP response elements and upon activation stimulates transcription at selected promoters (88). Transcription factors from the CREB/CREM family are required for cAMP-dependent proliferation in dog thyroid primary cultures and are tightly regulated by TSH and other factors (26). CREB activity has been shown to be important for TSH dependent thyrocyte proliferation in vitro and in vivo although it is not sufficient to mimic TSH-dependent DNA synthesis. In vitro, the importance of CREB was tested by stable transfection of FRTL-5 cells with either wild type CREB, which did not affect growth, or a dominant negative version dnCREB, which reduced up to 40% TSH-induced growth of the thyrocytes (100). In vivo, transgenic mice with targeted expression of dnCREB to their thyroid glands exhibited severe growth retardation and primary hypothyroidism; dnCREB inhibited the expression of the genes for Pax8, TTF-1, and TTF-2, which are required for the expression of TSHR, thyroglobulin and thyroperoxidase (101). Alterations of CREB family members can be observed in endocrine tumors (102), and levels of total CREB are markedly reduced in thyroid carcinomas (103), but their role in thyroid cancer remains highly controversial. Contradictory results have been reported in hyperfunctioning thyroid adenomas: some laboratories find a reduction of P-CREB (Ser 133) (104) while others do not find differences between nodular and extranodular tissue (103). Perhaps the interpretation of these results is too simplistic having in mind the multiple modifications that would affect CREB activity. It is generally believed that recruitment of the coactivators CREB-binding protein (CBP) and p300 after signal induced phosphorylation of CREB at Ser 133 strongly enhances its transcriptional activity. However, a number of kinases may phosphorylate CREB in Ser 133 including PKA, PKB/Akt, PKC, Rsk1/2. CREB activation can also be promoted by Ser 133 independent mechanisms and not all the signals that induce Ser 133 phosphorylation of CREB enhance its transcriptional activity. Furthermore, CREB is subjected to other phosphorylations, de-phosphorylations (by PP2A and PP1), acetylation by CBP, ubiquitylation and SUMOylation and, depending on its modifications, CREB interacts with other proteins that may perturb its localisation and/or turnover leading to activity changes [reviewed in (105,106)]. Thus CREB acts as a platform targeted by multiple signalling pathways where interference or integration takes place to render a gene expression profile extraordinarily tuned to incoming signals. CREB may use coactivators that are effectors of other signalling pathways (i.e. the Wnt effector β-catenin) and targets a number of genes involved in diverse aspects of proliferation, for example cyclin D1, which is also targeted by the Wnt signalling pathway and others. This provides new opportunities for integration. A complete genome wide analysis of CREB target genes has recently been published (107). Furthermore, other transcription (co)factors involved in proliferation such as β-catenin and others are also activated by PKA.

TSHR ALTERATIONS RELATED TO THYROID CANCER

Excesses or defaults in TSHR activity may play a role in thyroid disease and cancer. Both can be achieved by a number of mechanisms including: improper epigenetic marking of the gene, incorrect transcriptional regulation or mutations in critical domains.

Altered levels of TSHR expression

Lack or low TSHR expression correlates with aberrant methylation of the promoter in human thyroid carcinomas and in thyroid cancer cell lines (108). The silencing mechanism may also include the binding of the methylation-sensitive GABP transcription factor (23). TSHR function is required for NIS expression and iodide uptake. Low or absent TSHR expression and lack of NIS expression correlates with thyroid carcinomas of the worse prognostic that cannot be eliminated with radioactive iodide (109). Excessive TSHR expression does not correlate with malignant thyroid cancer to our knowledge, although a number of constitutively activating mutations have been described in thyroid adenomas as we describe below.

Mutations also change the levels of functional TSHR

Mutations may lead to altered location, turnover or recycling, activation/deactivation, postranslational

modifications or protein-protein interactions, including influences by and on other GPCRs, scaffolding, signaling mediators, desensitation, etc. TSHR activity is mainly controlled through protein-protein interactions and constitutively active mutants correlate with thyroid adenomas and nodules, while inactivating mutations correlate with diverse forms of hypothyroidism.

Inactivating mutations

More than 25 distinct loss-of-function mutations in the TSHR gene have been reported (41,110), including those occurring at the germ line and causing congenital hypothyroidism. So far none of these mutations appear to be related to the onset of thyroid cancer. Inactive TSHR fails to induce thyroid cell proliferation and is unlikely to induce nodule formation. Reduced TSHR expression in thyroid cancers may be secondary to ongoing de-differentiation, may happen in parallel or may cause disappearance of other differentiation markers.

The TSHR gene is highly susceptible to constitutively activating mutations

Structural studies discarded TSHR as a candidate oncogene for thyroid tumor (111), however G protein coupling is a critical step and most gain of function mutations are located in exons 9 and 10 corresponding to TM domains (112,113), ICLs or ECLs (114,115). Not surprisingly, these sites are critical for G protein coupling (see figure 3). A list of TSHR mutants may be found at <http://www.uni-leipzig.de/innere/tshr> and we have updated it in table 1. Spontaneous activating mutations of the TSHR gene appear at the onset of autonomous functioning thyroid adenomas (116,117) and more rarely of thyroid carcinomas (118-124). Somatic mutations cause autonomous nodules and germ-line mutations cause congenital hyperthyroidism and hereditary non-autoimmune toxic thyroid hyperplasia. Patients with activating TSHR mutations suffer hyperthyroidism; the disease activity directly correlates with the degree of TSHR activation measured as basal cAMP production (125). Likewise, thyroid nodules and goitre develop earlier in patients carrying TSHR variants with high constitutive receptor (126-130) [reviewed by (131)]. Furthermore, in multinodular goiters different TSHR activating mutations have been found in separate hot nodules of the same gland suggesting a role in true nodule formation (112). Constitutively activating mutations lie also at the root of toxic thyroid adenomas and differentiated thyroid carcinomas (120). Meanwhile, $G_{\alpha s}$ mutations are very infrequent in hyperthyroidism or toxic adenomas, suggesting that this subunit does not play an important role, a question intensely debated (132,133). Some authors have compared the characteristics and location of the most frequently appearing germ-line and somatic mutations in the TSHR gene. Germ-line mutations are mostly transitions and affect residues [183, 505, 509, and 597] never involved in somatic mutations. Somatic mutations are usually transversions and affect residues 630 and 633 never affected by germ-line mutations. Finally, several residues located in a mutation cluster region [619–639] at TM6 are affected by both somatic and germinal mutations (134).

Thus, the question of whether or not TSHR mutations are involved in the onset of thyroid cancers remains controversial as explained in previous sections. Interpretations are complicated by the fact that biological effects of activating TSHR mutations vary with the ambient iodide supply. In regions of iodine deficiency, higher incidence of toxic adenoma and toxic multi-nodular goitre has been reported, where 50–80% of these toxic adenomas are caused by TSHR mutations (135).

Postranslational modifications and protein interactions

Immunoelectron microscopy studies show that in human thyrocytes most of the TSHR is glycosilated and palmytoylated and is present on the cell surface. Other modifications such as acetylation, methylation, sumoylation, ubiquitination, etc. have not been reported, but interactions with several proteins that may scaffold its signalling or recycle back the endocytosed TSHR are important for its functionality. These TSHR interacting proteins include fibronectin, calreticulin, calnexin, β-arrestin, Scrib, HSP5, JAK1/2, Stat3, and other GPCRs such as the LHR. Mutations or altered expression in these protein effectors may give a phenotype related to thyroid cancer. For example, over expression of β-arrestin 2 has been found in thyroid toxic nodules (155). TSHR interacting proteins open new possibilities for networking the signals from the TSHR that will have to be explored. Besides the previously mentioned proteins other related hormones, such as hCG, or antibodies from autoinmune diseases, such as Graves' disease, may also interact with the TSHR and be involved in thyroid cancers. Anti-TSHR antibodies binding to the TSHR in thyroid nodules might promote growth via direct and indirect mechanisms such as upregulating the expression of insulin receptors (156), which may transduce IGF-II growth effects (157). Anti-TSHR antibodies also stimulate angiogenesis upregulating VEGF and its receptor (flt) in thyroid cells (158). Thus, high levels of anti-TSHR antibodies in Graves' patients might stimulate thyroid cancer growth and early metastatic spread [an issue that is discussed in (159)].

NETWORKING THE TSH RECEPTOR SIGNALLING IN THYROID CANCER

Cancer arises from accumulation of mutations in important genes or *oncogenes*. Most known oncogenes are key-signalling molecules important for the integrity of the signalling networks that maintain cell homeostasis. Oncogenes relevant to thyroid carcinogenesis are normally engaged in proliferation and/or survival pathways, the paradigms are RET/Ras/B-Raf, PTEN/Akt and E-cadherin/β-catenin representing the MAPK, PI3K and Wnt pathways respectively. These pathways are integrated in the thyrocyte signalling network among them and with the cAMP/PKA pathway and altering their crosstalk may lead to carcinogenesis.

The MAPK pathway and its cross talk with TSHR/cAMP/PKA

The MAPK pathway in the thyrocyte conveys proliferation signals from tyrosine kinase receptors such as RET. Hyperactivating RET mutations in the kinase domain define MTCs and rearrangements (RET/PTCs) are frequent at early onset of PTCs; RET mutants signal through B-Raf to activate the MAPK pathway in thyroid cells (160), and B-Raf mutations (V600E) are present in more than 40% of PTCs (161,162). The significance of the RET/Ras/B-Raf/ ERK pathway in thyroid carcinogenesis has been widely reviewed (163). Crosstalk between the Ras/ MAPK/ERK and the cAMP/PKA pathways has long been recognised and cAMP may either suppress or induce the MAPK pathway depending on the cell type (164). RET/PTC2 is a particularly interesting rearrangement cross-linking the MAPK and PKA pathways because the kinase domain of RET is fused to the regulatory subunit of PKA II (165). In FRTL-5 rat thyrocytes, both TSH and cAMP induce ERK (166), and these cells require Ras for TSH-stimulated mitogenesis (90,91); in dog and human thyrocytes, indirect evidence suggest the same because a MEK inhibitor blocks TSH stimulated DNA synthesis (167). cAMP may activate B-Raf and ERK through PKA-dependent or independent mechanisms that need further clarification at the thyrocyte. Thus, in thyroid cells cAMP does not inhibit MAPK signalling but whether it activates it or not remains controversial due to differences among distinct in vitro systems (15). The high sensitivity to B-Raf mutations exhibited by thyrocytes in cancer is intriguing and remarkable and perhaps is exacerbated by the lack of cAMP-dependent mechanisms to suppress B-Raf/ERK activity.

The PI3K/AKT pathway and its crosstalk with TSHR/cAMP/PKA

PI3K/AKT is a pleiotropic kinase downstream of many growth factor receptors involved in cell survival, proliferation and cancer. Indirect evidence of the role of PI3K in thyroid cancer comes from the use of an Akt inhibitor (KP372-1) that suppresses proliferation and induces apoptosis in thyroid cancer cells (168). Activating mutations of Akt or silencing its upstream suppressor PTEN (Phosphatase and TENsin homolog) is frequently associated with thyroid carcinoma (169). PTEN is a dual specificity phosphatase mutated or silenced in the majority of human advanced cancers. Also in thyroid cancers has been found loss, reduction, or inappropriate subcellular compartmentalization of PTEN (170,171). A rearrangement with the histone H4 gene has been found in irradiated thyroid cell lines, H4/PTEN (172). PTEN silencing has been suggested to be involved in the carcinogenesis of highly malignant or late-stage thyroid cancers (170,173), and reintroduction of PTEN in thyroid cancer cell lines causes G1 arrest in the differentiated and apoptosis in the most undifferentiated thyroid cancer cell lines (174). TSHR immunoprecipitates exhibit PI3K activity, which is greater after TSH treatment. Concomitantly, the kinase PDK1 is redistributed from the cytoplasm to the plasma membrane in a PI3K- and PKA-dependent manner after TSH treatment (175). The regulatory subunit of PI3K, p85 phosphorylated at Ser 83, binds the regulatory subunit of PKA, RIIβ and mediate TSH-cAMP-PKA growth and survival signals (176). Moreover, the PI3K/Akt pathway has been suggested to be activated in thyroid tumors by RET oncoproteins that associate with RAI (ShcC/N-Shc) and recruit GAB 1 (Grb 2-associated binder 1) (177). Additionally, PI3K is involved in IGF-1 or insulin mediated cooperation with TSH for DNA synthesis in thyrocytes. The interactions between the Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt pathways are crucial to regulate growth and may be altered in tumorigenesis.

The Wnt pathway

The Wnt pathway plays a pivotal role in development and in epithelial renewal and components such as β-catenin, APC and E-cadherin are often mutated in thyroid cancer (178,179). β-catenin mutations are more frequent in undifferentiated cancers or ATCs (180,181), and abnormalities of the E-cadherin/catenin complex (methylation of the promoter and silencing or mutations) are associated with the more aggressive behaviour of certain PTCs (182). The Wnt components E-cadherin and β-catenin may crosstalk with the classical TSH/cAMP/CREB pathway to con-

trol thyrocyte proliferation. PKA is known to potentiate Wnt signalling in some cells stabilizing β-catenin and inhibiting GSK3β (183,184). The CREB transcription factor might be targeted by both PKA and Wnt signalling, and recently its role in lithium-stimulated thyrocyte proliferation has been challenged (185). Although these authors conclude that CREB does not play a role in lithium-induced thyroid proliferation, their experiments do not rule out a possible role of CREB in Wnt-dependent proliferation of thyrocytes. Some components of the Wnt pathway (GSK3β) are also targeted by PI3K. Cyclin D1 is a transcriptional target for both CREB and β-catenin and is over expressed in thyroid papillary microcarcinoma with aberrant β-catenin (186).

In summary, mutations of individual components may cause fundamental functional changes well beyond the pathway they function in, due to the crosstalk between signalling pathways. One of the molecules most mutated in cancer in general and in ATC also is p53, another common target for many signalling pathways including PI3K, cAMP, and ERK. p53 is inactivated in 50% of human cancers and 14% of malignant thyroid tumors; p53 mutations appear "late" in thyroid carcinogenesis and are associated with loss of differentiation and transformation to the anaplastic phenotype (132). p53 is a transcription factor and plays multiple regulatory functions in the cell cycle, DNA repair, and apoptosis. Reintroduction of wild type p53 in thyroid tumoral cell lines arrests growth and/or induces apoptosis (21,187). p53 protein levels may be increased by cAMP, ERK, and also through the activation of PTEN, and recently a differential proteomic approach has identified several targeted proteins for mutant p53 associated with thyroid cell transformation (188). The TSHR and p53 connection in thyrocytes still has to be clarified but it is remarkable that stable expression of a p53 mutant which does not bind DNA (V143A), induces loss of differentiation markers and TSH-independent growth in PC CL3 thyrocytes. In contrast, a p53 mutant (S392A), which does not interfere with DNA binding, causes only loss of TSH dependency for growth (188).

FUTURE PERSPECTIVES

Undifferentiated thyroid cancers are among the most rapid life-threatening and are characterised by abnormal CpG islands methylation patterns in critical promoters. Silencing of TSHR, PTEN and p53 appears to be related to the most aggressive behaviour of thyroid cancer, and reintroduction of each of these genes in human thyroid cancer cell lines either restores differentiation or arrest cell cycle or causes apoptosis. Hypomethylation of heterochromatin has been correlated with tumour progression in thyroid cancer specimens (189), and hypermethylation of tumor supressor promoters appears in undifferentiated thyroid cancer causing failure in clinical radioiodine treatment (171,173). Thus, aberrant DNA methylation patterns are some of the epigenetic marking processes more likely involved in the onset of thyroid cancer. Demethylating agents or inhibitors of the DNA methylase may be promising hopes in the treatment of the worse thyroid cancers, but their clinical value remains to be investigated and they have to be specifically targeted. In general, the development of molecular-based therapies for thyroid carcinoma patients resistant to standard radioiodine treatment such as histone deacetylase inhibitors combined with retinoids or the use of specific targeted RNAis for invasion/metastasis molecules represent a critical issue and an exciting field in new drug development in thyroid cancer.

Large chromatin alterations related to thyroid cancer, such as the chromosomal rearrangements: RET/PTCs or Pax8/PPARγ chimeras (190), coincide in their activation of the Ras/Raf/MEK/ERK pathway, which is also induced by TSHR signalling in the thyrocyte. Chromatin alterations related to cancer, especially epigenetic modifications, need to be further explored, especially lost of imprinting (LOI) and lost of heterozigosity (LOH) studies will reveal new clues to the onset of cancer.

Finally, an exciting aspect in thyroid cancer research is the definition and biological characterization of the precursor cells that give rise to thyroid carcinomas. Mouse embryonic stem cells might now be differentiated into thyroid follicular cells in vitro (5), and this system will surely help to identify key events in thyroid carcinogenesis and understanding the role of TSHR.

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