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Mouse models of follicular and papillary thyroid cancer progression

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A significant number of well-differentiated thyroid cancers progress or recur, becoming resistant to current therapeutic options. Mouse models recapitulating the genetic and histological features of advanced thyroid cancer have been an invaluable tool to dissect the mechanisms involved in the progression from indolent, well differentiated tumors to aggressive, poorly differentiated carcinomas, and to identify novel therapeutic targets. In this review, we focus on the lessons learned from models of epithelial cell-derived thyroid cancer showing progression from hyperplastic lesions to locally invasive and metastatic carcinomas.

Keywords: thyroid, cancer, mouse, progression

INTRODUCTION

The thyroid gland is the most common target of neoplastic transformation among endocrine organs. Contrary to most tumor types, the global incidence of thyroid cancer has been steadily growing in recent years. Most thyroid cancers derive from the thyrocytes, the epithelial cells that delineate the thyroid follicles, and include well-differentiated tumors, such as papillary and follicular carcinomas, as well as poorly differentiated and anaplastic carcinomas. Although well-differentiated thyroid cancers have generally a good prognosis and can be effectively managed through a combination of surgery and radioactive iodine treatment, a number of them will progress or recur, becoming resistant to current therapeutic options. On the other hand, poorly differentiated and anaplastic tumors often derive from pre-existing well-differentiated lesions and are responsible for the vast majority of thyroid cancer-related deaths, thanks to their very poor response to therapy.

In order to design rational approaches to these otherwise unresponsive tumors, we need to better define and understand the molecular mechanisms leading to progression from indolent, well-differentiated tumors to aggressive cancers. One strategy to accomplish this task is to generate model systems that more or less faithfully recapitulate *in vivo* what has been learned from molecular pathology studies on patient material. These models can then be used to dissect in a physiological setting the pathways deregulated as a consequence of specific genetic alterations.

Advances in the genetic manipulation of the mouse genome have facilitated the generation of models harboring defined genetic alterations that can be controlled both temporally and spatially. Although their use in preclinical studies is often hampered by the time needed to develop tumors, by the generally low metastasis incidence, and by some biological differences between mice and humans in drug metabolism, mouse models of cancer have been an invaluable tool to study gene function, interaction between pathways, and the specific mechanisms that play a role in tumor progression.

In this review, we will focus on what we have learned from mouse models of thyroid cancer that display progression from hyperplastic lesions to locally invasive and metastatic carcinomas.

PTC PROGRESSION MODELS

Papillary thyroid carcinoma (PTC) is the most common subtype among differentiated thyroid tumors and represents up to 80% of all malignant thyroid cancers. Patients affected by PTC are effectively treated with thyroidectomy followed by radioactive iodine ablation (DeGroot et al., 1990). After surgery the survival rate and the therapeutic outcomes of PTC patients are favorable. However, up to 10% of PTC patients can exhibit lymph node metastases or distant metastases at the time of thyroid ablation that are associated with a significant increase in morbidity (Huang et al., 2011). The etiology of papillary carcinomas is often correlated with a diet poor in iodine and with radiation exposure. In particular there are evidences that survivors of cancers of various organs whose tumor had been treated with radiation developed, later on in their life, papillary thyroid cancers (LiVolsi, 2011). Older age, male sex, large size, and extrathyroidal extension can be a contributing

factor for a negative prognosis, although they are not entirely reliable in predicting tumor recurrence, metastasis, and cancer-related death. The diagnosis for PTCs is based on conventional tumor morphology and on the WHO histological classification (Kakudo et al., 2011). This classification is based on nuclear features such as ground glass appearance, pale staining, inconspicuous nucleoli, large size, and irregular outline together with the presence of vascular core, psammoma bodies, fibrous stroma, squamous metaplasia, and presence of follicles (LiVolsi and Baloch, 2004). A minority of PTCs progress from well-differentiated carcinoma to either poorly or undifferentiated carcinoma, an event associated with a marked reduction in survival. However, whether certain subtypes of papillary carcinoma are associated with a more aggressive clinical behavior is still unclear. Moreover the invasive features are often difficult to predict only from the histological analysis. There is increasing evidence suggesting that PTCs represents a rather heterogeneous group of tumors, and that specific genetic alterations may not only determine the distinct global expression profile of the tumor, but also influence its histologic variants and tumor behavior (Giuffrida and Gharib, 2000).

Over the past decade, the genetic and epigenetic basis for PTCs has been defined and distinct activating mutations of oncogenes have being described together with epigenetic silencing of tumor suppressor genes (Kimura et al., 2003). The earliest, most common, and best-characterized somatic genetic alterations in PTCs are the *RET/PTC* rearrangement (Santoro et al., 1993), the *TRK-T1* mutation, and the $BRAF^{V600E}$ point mutation. They appear to be mutually exclusive, but it has been demonstrated that they work along the same signaling cascade that starts with the phosphorylation of RET on tyrosine 1062, and sequentially triggers RAS, BRAF, and ERK stimulation. Moreover the three activated oncoproteins induce largely overlapping gene expression signatures (Melillo et al., 2005).

The acquisition of a migratory phenotype and the loss of tumor suppressor genes such as *TP53*, lead to progression toward poorly differentiated (PDTC), and undifferentiated thyroid carcinoma (ATC; Giordano et al., 2005).

Transgenic mice carrying the aforementioned mutations have been instrumental in increasing our understanding of the mechanisms that are responsible for the progression from PTC to PDTC or ATC.

RET/PTC MODELS

RET/PTC mutations are frequently detected in PTC, particularly in radiation-induced PTC. The *RET/PTC* rearrangement is the second most common type of genetic alteration known to occur in thyroid papillary carcinomas (Pierotti et al., 1996).

The *RET* proto-oncogene encodes a transmembrane glycoprotein belonging to the receptor tyrosine kinase (RTK) family. It is the receptor for growth factors belonging to the glial cell line-derived neurotrophic factor (GDNF) family. The fusion of the 3′-terminal kinase-encoding domain of *RET* to the 5′-terminal regions of heterologous genes leads to the formation of a chimeric oncogene. There are at least 15 different *RET/PTC* rearrangements but *RET/PTC1* and *RET/PTC3* are the most prevalent variants. *RET/PTC1* originates from the fusion between *RET* and the *H4* gene (Grieco et al., 1990), while *RET/PTC3* originates from the *RET-RFG* (also designed as *ELE1*, *NCOA4*, *ARA70*) fusion (Santoro et al., 1994). All the other rearrangement variants have been isolated just in rare cases (Fusco and Santoro, 2007).

While the *RET* proto-oncogene products localize in the membrane fraction and are not phosphorylated in the absence of a ligand, the *RET/PTC* product localizes in a soluble cytoplasmic fraction and is constitutively phosphorylated (Ishizaka et al., 1992). RET triggers several intracellular signaling cascades, which regulate survival, differentiation, proliferation, migration, and chemotaxis through the activation of targets such as the Ras/ERK kinase, PI3 kinase/AKT, p38MAP kinase, PLC-γ, JNK, STAT, ERK5, and Src signaling pathways (Sariola and Saarma, 2003).

RET/*PTC3* is more efficient than *RET/PTC1* in promoting proliferation of cultured thyroid cells. Accordingly, transgenic mice expressing the human *RET/PTC1* gene under the control of the bovine thyroglobulin promoter develop PTCs, demonstrating that *RET/PTC* oncogenes are able to initiate thyroid carcinogenesis (Santoro et al., 1996). However, invasive cancer does not occur in these models. On the other hand, transgenic mice expressing the human *RET/PTC3* gene under the control of the same bovine thyroglobulin promoter develop papillary carcinoma with metastatic spread in selected cases (Jhiang et al., 1998; Powell et al., 1998). The severity of disease varies markedly between high- and low-copy number founder lines. High-copy lines have dysplastic thyroid glands at birth and develop carcinomas as young as 4 days of age, as compared with 1–6 months of age in the low-copy line (Cho et al., 1999).

RET/PTC1 mice maintained on a low iodine diet present a persistent elevation of thyroid-stimulating hormone (TSH) levels and enhanced tumorigenesis. However, the high TSH levels of the *RET/PTC1* transgene are a disadvantage of this model, because chronic TSH stimulation is generally not involved in papillary carcinoma development in humans (Williams et al., 2004), although recent data seem to suggest a correlation between TSH levels and tumor size (Zafon et al., 2012).

One possibility that could explain the limited ability of small tumors with RET gene rearrangements to progress, is the pro-apoptotic activity of RET/PTC. It has been shown that during tumor progression, secondary events, such as overexpression of the anti-apoptotic protein Bcl-2 or activation of other cell survival pathways (Gimm et al., 2001), including overexpression of AKT or downregulation of *PTEN*, can be selected to suppress RET/PTC-induced apoptosis and to promote the expansion of the neoplastic clones (Castellone et al., 2003).

An additional event that could suppress apoptosis and allow tumor progression is loss of p53. In general, lack of functional p53 protein alone is not sufficient to induce a malignant phenotype (Battista et al., 1995), however, p53 loss in the presence of *RET*/*PTC1* mutation contributes to the progression and metastasis formation of thyroid epithelial neoplasms. Transgenic mice expressing *RET/PTC1* under the control of the bovine or rat thyroglobulin promoters in a $p53^{-/-}$ background develop anaplastic-like tumors. These tumors are bilateral, and TSH-responsive, with cystic and solid regions. The solid regions are composed of spindle cells, which is a common feature of ATCs in human (La Perle et al., 2000). One caveat of this model is that compound mutant

mice often died because of extrathyroidal tumors, due to the "non-tissue-specific" p53 mutation. Unfortunately a thyroid-restricted *RET/PTC1*, $p53^{-/-}$ mouse model does not exist yet. The generation of this mouse model would likely allow the mice to live enough for metastases to develop.

The more aggressive *RET/PTC3* transgenic mice develop follicular cell hyperplasia and a solid variant of PTC by the age of 6 months (Powell et al., 1998). Less than 10% of these mice develop metastases to cervical lymph nodes. Interestingly, *RET/PTC3* thyroids expressed proinflammatory genes and cytokines such as Cox-2, Gmcsf, Il1α, Il1β, Mcp1, Il6, and Tnfα, and recruited macrophages to the lesions, a process known to aid tumor progression (Russell et al., 2004). The *RET/PTC3*-induced thyroid tumors do not dedifferentiate into a more aggressive phenotype as often as seen in human patients with *RET/PTC3* chromosomal rearrangements. Intercross of Tg-*RET/PTC3* transgenic mice with $p53^{-/-}$ mice results in earlier tumor onset, with microcarcinoma formation by 3 months of age and the development of larger primary tumors compared to the *p53* wild-type mice. However metastases are still rare (Powell et al., 2001).

TRK-T1 MODELS

The TRK family of oncogenes results from a genetic fusion of the 3′ end of *NTRK1* to the 5′ end of several partner genes (Pierotti et al., 1996). In particular the *TRK* oncogene results from the inversion of chromosome 1 that joins the 5′ end of Tropomyosin (*TPM3*) to *NTRK1* (Bongarzone et al., 1989). *TRK-T1*, *-T2*, and *-T4* are generated from inversions of chromosome 1 that join the 5′ end of *TPR* to the 3′ end of *NTRK1*. A rearrangement involving the genes *TFG* and *NTRK1* results in the family member *TRK-T3*.

NTRK1-related translocations have a reported incidence of about 12%. *In vitro*, the TRK-T1 oncogene transforms NIH3T3 cells (Greco et al., 1992). The only described mouse model of NTRK1 rearrangement is a transgenic model of *TRK-T1* under the control of the bovine thyroglobulin promoter. More than 50% of the *TRK-T1* mice show thyroid epithelial cell hyperplasia which progresses to PTCs in animals over 7 months of age, and has the histopathological features observed in its human counterpart.

Few thyroid tumors in *TRK-T1* mice developed less-differentiated solid regions during 24 months of observation, and none of the tumors metastasized. It is clear, as it happens in *RET/PTC* mice, that while *TRK-T1* predisposes mice to develop PTCs, additional genetic mutations are required to evolve into more aggressive tumors (Russell et al., 2000). Thus, the *TRK-T1* fusion gene may allow thyroid follicular epithelial cells to survive and accumulate other genetic abnormalities. A representative example of this concept is the cross between *TRK-T1* mice and $p27^{-/-}$ mice. Decreased expression and altered subcellular localization of p27 have been observed in thyroid cancer (Motti et al., 2005) and a correlation has been found between the overexpression of the *TRK-T1* transgene and *p27* deletion in thyroid carcinomas. The *p27* mutation increases the malignant phenotype of mice expressing the *TRK-T1* oncogene: the compound mutants show an increased number of carcinomas with a shorter latency and an increased proliferation rate (Fedele et al., 2009).

$BRAF^{V600E}$ MODELS

BRAF is a member of the RAF kinase family. The *BRAF* gene, encoding a cytosolic serine–threonine protein kinase, serves as an immediate downstream effector of RAS. BRAF transmits signals from RAS to the mitogen-activated protein kinase (MAPK) pathway through MEK and ERK. Over 30 *BRAF* mutations associated with human cancers have been described, and the majority is located within the kinase domain. Common *BRAF* somatic activating mutations have been identified in melanomas (70% prevalence), and colorectal and ovarian cancers (15%), as well as in thyroid cancers (>40% papillary carcinomas). *BRAF* mutations are particularly common in melanomas and thyroid cancer. The growth of melanocytes and thyrocytes is positively regulated by cAMP. In particular cAMP activates MEK1 and ERK through mechanisms that converge on BRAF. Thus, BRAF is the key RAF isoform transducing cAMP-dependent growth signal in both these cell types (Busca et al., 2000), and this, together with the higher basal activity and simpler activation mechanism of BRAF compared to ARAF and CRAF (Maurer et al., 2011), can reasonably explain their vulnerability to transformation by *BRAF* activating mutations (Kimura et al., 2003).

The *BRAF* mutation found in the vast majority of thyroid cancers is a thymine-to-adenine transversion at nucleotide 1796 (T1796A), resulting in a valine-to-glutamic acid substitution at amino acid 600 (V600E; Fukushima et al., 2003). *BRAF* point mutations are found at all stages of progression, from microcarcinomas to poorly differentiated and anaplastic carcinomas, suggesting that this mutation is responsible for the initiation of the transformation process.

Thyroid-specific expression of $BRAF^{V600E}$ induces goiter that progresses to invasive PTC with tall-cell features. After 22 weeks of age, mice develop aggressive thyroid papillary carcinomas with a high frequency of invasion into blood vessel, thyroid capsule, and skeletal muscle (Knauf et al., 2005). *BRAF*-mutant papillary carcinomas have a high degree of lymphocytic infiltration within the tumor, but less frequent psammoma bodies as compared to tumors harboring *RET/PTC* rearrangements. Furthermore, *BRAF*-mutant tumors have a different gene expression profile, compared to RET/PTC tumors, with a significant deregulation of genes related to the innate immune response. There is a strong correlation between increased tumor-associated macrophages (TAMs) and histologic grade, and between TAMs, tumor invasiveness, and decreased cancer-related survival in PDTC. $BRAF^{V600E}$ mice indeed show a high density of TAMs, suggesting that TAMs promote papillary thyroid cancer progression (Ryder et al., 2008).

The $BRAF^{V600E}$ mutation induces also the expression of genes involved in matrix remodeling, which could account in part for the greater predisposition of PTC with *BRAF* mutation to invade surrounding tissues (Mesa et al., 2006). In human patients, PTCs harboring $BRAF^{V600E}$ show a more aggressive clinicopathologic behavior, with extrathyroidal extension and a significant increase in MMP-2, MMP-9, and ICAM-1 protein levels, suggesting that these proteins may play a role in PTC progression (Frasca et al., 2008). Furthermore, high levels of CXCR4 expression were found in $BRAF^{V600E}$-positive PTCs, and CXCR4 expression was associated with thyroid capsule infiltration and extrathyroidal

extension (Torregrossa et al., 2011). These findings await in-depth mechanistic analysis in the *BRAF*^{V600E} mouse model.

Finally, an expression profile study of murine PDTCs derived from *BRAF*^{V600E} transgenic mice showed an unequivocal signature of epithelial-to-mesenchymal transition (EMT; Knauf et al., 2011). EMT involves changes in epithelial cells that disassemble their junctional structures, express mesenchymal proteins, remodel their extracellular matrix, lose polarity, and become more migratory. Thus, this model will allow a more mechanistic approach to the problem of thyroid cancer dedifferentiation and progression to poorly differentiated and anaplastic tumors.

Although this first mouse model of *BRAF*^{V600E}-positive PTC recapitulates the phenotype of human PTCs, it displays severe hypothyroidism that is not usually present in humans. A recent paper describes a novel inducible PTC model (*Thyro::CreER*^{T2}; *BRaf*^{CA}) in which widespread Cre-induced BRAF activation generates the same tumor and hypothyroid phenotype as the older model. However, stochastic tamoxifen-independent Cre activity induces localized lesions without concurrent hormonal derangement. Therefore this model may be, in some aspects, a more accurate model of human PTC (Charles et al., 2011).

The recent development of novel small-molecule inhibitors targeting BRAF^{V600E} may provide selective and rational advantages for the treatment of patients with PTC harboring this mutation. In particular, two Plexxikon compounds, PLX4720 and PLX4032, are novel, orally available selective small-molecule inhibitors of BRAF^{V600E} that have been specifically designed to insert into the ATP-binding site and trap oncogenic BRAF^{V600E} in an inactive conformation. These compounds have shown an important effect in a cohort of melanoma patients (Bollag et al., 2010).

In thyroid tumors, *in vitro* treatment of *BRAF*^{V600E}-positive thyroid cancer cell lines with PLX4720 resulted in cell cycle arrest, whereas *RET/PTC1* and *RAS* mutated thyroid cancer cell lines were resistant to inhibition even at high concentrations (Salerno et al., 2010). Furthermore, PLX4720 markedly inhibited tumor growth and metastasis in an orthotopic mouse model of human ATC harboring *BRAF*^{V600E} (Nucera et al., 2011). PLX4032 is being tested in human *BRAF*^{V600E} carriers. Early results show one partial response and two patients with a brief stable disease among the three PTC patients analyzed in a phase I trial (Flaherty et al., 2010). Accordingly, evidence is accumulating that *BRAF*^{V600E} tumors rapidly develop resistance to BRAF inhibitors (Nazarian et al., 2010). *In vivo* studies using the aforementioned mouse models will shed more light on the mechanisms implicated in resistance development, and on the possibility of overcoming resistance by targeting the pathways involved.

FTC PROGRESSION MODELS

Follicular thyroid carcinoma (FTC) represents the second largest subtype of thyroid malignancies, and is more prevalent in areas of dietary iodine deficiency (Schlumberger, 1998). FTC patients have a poorer outcome than those affected with PTC since they are less likely to take up radioactive iodide for imaging and therapeutic ablation (Jonklaas et al., 2006). Histologically, FTCs display variable morphology ranging from small/medium-sized follicles containing colloid to trabecular or solid growth pattern (Schlumberger, 1998). It is extremely rare for a FTC to be composed of macrofollicles (Fonseca et al., 2006).

The major molecular features of FTC are the prominence of aneuploidy and the high prevalence of *RAS* mutations and of *PAX8–PPARγ* rearrangements, the latter as a result of a chromosome 2;3 translocation (DeLellis, 2004). The *PAX8–PPARγ* rearrangement leads to in-frame fusion of exon 7, 8, or 9 of *PAX8* on 2q13 with exon 1 of *PPARγ* on 3p25 (Kroll et al., 2000). This translocation creates a fusion gene composed by the DNA-binding domain of the thyroid transcription factor *PAX8* and domains A to F of the nuclear receptor *PPARγ* (Eberhardt et al., 2010). The exact mechanism by which this rearrangement leads to a carcinogenic phenotype is not fully understood. It appears as though the PAX8–PPARγ chimeric protein inactivates the wild-type PPARγ, which is a putative tumor suppressor (Suh et al., 1999; Chang and Szabo, 2000).

Follicular neoplasms are also often characterized by PI3K/AKT alterations. Phosphorylation of AKT, the key player in this pathway, is far more frequent than that of ERK in FTCs (Xing, 2010). The aneuploid pattern and the various molecular alterations detected in FTCs support the assumption that the development of such tumors is the endproduct of multiple oncogenic steps, thus justifying their usual appearance as single neoplasms (Fagin, 2002).

Most models for FTC were established based on the genetic alterations described in human tumors such as *Ras* mutations and *PAX8–PPARγ* rearrangement. However, mouse models based on these genetic alterations have failed to fully recapitulate human FTC. Instead, genetically engineered models carrying a thyroid hormone receptor β mutant (TRβ$^{PV/PV}$) and deletion of the *Pten* tumor suppressor have become the tool of choice to study the development of FTC.

TRβ$^{PV/PV}$ MODELS

Thyroid hormone receptors (TRs) are ligand-dependent transcription factors that have a central role in cell proliferation, differentiation, and apoptosis. They are members of the steroid hormone/retinoic acid nuclear receptor superfamily. Two genes, *TR*α and *TR*β, located on human chromosome 17 and 3, respectively, encode for four thyroid hormone (triiodothyronine T3) binding receptors (TRα1, TRβ1, TRβ2, and TRβ3) and two non-T3-binding receptors (TRα2 and TRα3). These TRs are expressed in a tissue-dependent and developmentally regulated pattern (Cheng, 2000). Mutations of the TRs can cause a potent dominant negative action (Lin et al., 1999). Furthermore, multiple mutations in both TRα1 and TRβ1, with impairment in T3 and DNA binding and loss of transcriptional activity, were detected in human renal clear cell carcinoma (Kamiya et al., 2002). Unusually high frequencies of mutations in TRβ1 and TRα1 were also found in a subset of human PTC (93.75 and 62.25%, respectively, of 16 tumors examined; Puzianowska-Kuznicka et al., 2002).

Kaneshige et al. (2000) generated a mutant knock-in mouse expressing a potent dominant negative *TRβ1* mutant, *PV* (*TR*β^{PV} mice). The *PV* mutation was identified in a patient with thyroid hormone resistance syndrome (RTH; Parrilla et al., 1991; Weiss and Refetoff, 2000). RTH is a genetic disease caused by mutations

of the *TR*β gene (Weiss and Refetoff, 2000). Most patients with RTH are heterozygotes with only one mutant *TR*β allele (Weiss and Refetoff, 2000). Patients with RTH exhibit dysfunction of the pituitary–thyroid axis, with high circulating levels of TSH and thyroid hormones (T3 and thyroxine T4; Weiss and Refetoff, 2000). PV has a unique mutation in exon 10, a C-insertion at codon 448, which causes a frameshift of the carboxy-terminal 14 amino acids of TRβ1, resulting in total loss of T3-binding and transcriptional activation (Meier et al., 1992).

TRβ^{PV} mice faithfully reproduce human RTH because they exhibit mild disruption of the thyroid–pituitary axis and a severe dysfunction of the pituitary–thyroid axis (Kaneshige et al., 2000). In addition, *TR*$\beta^{PV/PV}$ homozygotes develop follicular cell hyperplasia by 3 weeks of age and invasive FTCs by 4–5 months of age. They also display metastases to the lungs and heart by 5 months of age, and the metastases often have foci of anaplasia consisting of spindle-shaped cells (Suzuki et al., 2002). This mouse model offers a fairly good recapitulation of human follicular carcinoma arising from hyperplastic goiter, but it differs in that these mice have elevated circulating T3 and T4 levels, while human FTCs are infrequently functional (Kaneshige et al., 2000).

Kato et al. (2004) crossed *TR*β^{PV} mice with *TR*$\beta^{-/-}$ mice. As *TR*$\beta^{PV/-}$ mice aged, they also spontaneously developed FTC after progression from hyperplasia, with local invasion, anaplasia, and metastasis to the lungs (Kato et al., 2004). The pathological progression of thyroid carcinoma in *TR*$\beta^{PV/-}$ mice was identical to that in *TR*$\beta^{PV/PV}$ mice, and the patterns in the alteration of signaling pathways were also similar to those observed in *TR*$\beta^{PV/PV}$ mice during thyroid carcinogenesis (Kato et al., 2004). These results point out that *TR*β may function as a tumor suppressor, since, in the absence of a wild-type allele, the *PV* mutation in one *TR*β allele is sufficient for the mutant mice to spontaneously develop FTC.

One of the overexpressed genes in mice carrying the *TR*$\beta^{PV/PV}$ genotype is the pituitary tumor-transforming gene (PTTG), which has been previously associated with tumorigenesis (Ramaswamy et al., 2003). To evaluate the role of PTTG in thyroid carcinogenesis, Kim et al. (2007b) analyzed the offspring of *TR*β^{PV} mice crossed with mice lacking PTTG (*PTTG*$^{-/-}$mice). The thyroids of *TR*$\beta^{PV/PV}$ *PTTG*$^{-/-}$ mice resulted significantly smaller than those of *TR*$\beta^{PV/PV}$ mice, with a decrease in thyroid proliferation. Histological analysis showed no difference in FTC occurrence between *TR*$\beta^{PV/PV}$ and *TR*$\beta^{PV/PV}$; *PTTG*$^{-/-}$ mice, which indicates that *PTTG* deletion does not prevent the initiation of FTC. However, *TR*$\beta^{PV/PV}$, *PTTG*$^{-/-}$ mice exhibited a significant decrease in vascular invasion and less development of lung metastasis as they aged. CD31 staining also showed a decrease in vessel density in *TR*$\beta^{PV/PV}$; *PTTG*$^{-/-}$ versus *TR*$\beta^{PV/PV}$ thyroids, highlighting the dual roles of PTTG as a regulator of thyroid growth and contributor of tumor progression (Kim et al., 2007b).

Kim et al. (2007a) also tested the role of gelsolin, an actin-regulatory protein, which had been previously found down-regulated in the mutant mice and in many other cancers such as breast (Winston et al., 2001), bladder (Tanaka et al., 1995), lung (Dosaka-Akita et al., 1998), and prostate (Lee et al., 1999). The results of these studies showed that there was an age-dependent reduction of gelsolin protein abundance in *TR*$\beta^{PV/PV}$ mice as tumorigenesis progressed. Knockdown of gelsolin by small interfering RNA resulted in increased tumor cell motility whereas an increase in gelsolin expression led to decreased cell motility (Kim et al., 2007a). Further biochemical analysis demonstrated that gelsolin physically interacts with the DNA-binding domain TRβ1 or PV *in vivo* and *in vitro*, and that the interaction with PV reduces gelsolin's binding to actin, leading to disarrayed cytoskeletal architectures (Kim et al., 2007a). These results suggest that PV-induced alterations of the actin/gelsolin cytoskeleton contributes to increased cell motility that contributes to the local tumor progression and metastatic potential of thyroid carcinogenesis.

Since the *TR*$\beta^{PV/PV}$ mice exhibit highly elevated TSH levels, Lu et al. (2010) crossed the *TR*$\beta^{PV/PV}$ mice with *TSHR* knockout mice (*TSHR*$^{-/-}$ mice) to eliminate the effect of TSH–TSHR signaling during carcinogenesis. They found that the thyroid of *TR*$\beta^{PV/PV}$ *TSHR*$^{-/-}$ mice exhibited impairment in growth and did not develop tumors. However, TSH signaling, while necessary, was not sufficient for transformation, since wild-type mice treated with the goitrogen PTU had a 9.1-fold higher serum TSH level than *TR*$\beta^{PV/PV}$ mice, but the degree of thyroid pathology was less than that of *TR*$\beta^{PV/PV}$ mice at the same age (Lu et al., 2010).

The PI3K/AKT pathway is activated in both the thyroid and metastatic lesions of the *TR*$\beta^{PV/PV}$ mouse model (Kim et al., 2005). Since the activation of the PI3K/AKT signaling cascade contributes to thyroid carcinogenesis, this pathway becomes a potential therapeutic target in FTC. When the *TR*$\beta^{PV/PV}$ mice were treated with LY294002 (LY), a potent PI3K inhibitor, a decrease in cyclin D1 expression was observed (Furuya et al., 2007). Concomitantly, the mice displayed an increase in $p27^{Kip1}$ expression to inhibit thyroid tumor growth and reduce tumor cell proliferation. LY treatment increased caspase-3 and decreased phosphorylated-BAD to induce apoptosis. In addition, LY treatment reduced the AKT–matrix metalloproteinase 2 signaling with a decrease in cell motility to block metastatic spread of thyroid tumors (Furuya et al., 2007). Therefore, this treatment effectively prolonged the survival of *TR*$\beta^{PV/PV}$ mice, providing the first preclinical evidence of the *in vivo* efficacy of PI3K inhibition in the treatment of FTC.

More recently, tumor development, progression, and metastasis were analyzed in the *TR*$\beta^{PV/PV}$; *Akt1*$^{-/-}$ mouse model (Saji et al., 2011). PV-dependent thyroid hyperplasia occurred in both groups *Akt1* WT and KO mice; however, the thyroid size was greater in the WT mice. On the other hand, thyroid cancer development was delayed in the KO mice and the degree of tumor invasiveness was reduced (Saji et al., 2011). The WT mice displayed pulmonary metastases at 12 and 15 months of age, while KO mice did not. In spite of sustained expression of Akt2 or Akt3, pAkt levels were decreased, suggesting that thyroid cancer development and progression in *TR*$\beta^{PV/PV}$ mice are Akt1-dependent.

Along the same pathway, to assess how *Pten* deregulation contributes to the PV phenotype, *TR*$\beta^{PV/PV}$, *Pten*$^{+/-}$ mice were generated and analyzed (Guigon et al., 2009). These studies demonstrated that *Pten* deficiency accelerated the progression of thyroid tumors and increased the occurrence of metastases to the lungs in the PV mice, thereby significantly reducing their survival as compared with *TR*$\beta^{PV/PV}$, *Pten*$^{+/+}$ mice (Guigon et al., 2009). AKT activation was concomitantly increased by twofold in

TRβ$^{PV/PV}$, *Pten*$^{+/-}$ thyroids, leading to increased activity of the downstream mammalian target of rapamycin (mTOR)–p70S6K and decreased activity of the forkhead family member FOXO3a. In addition, cyclin D1 expression was increased, and apoptosis was decreased as indicated by increased expression of nuclear factor-κB (NF-κB) and decreased caspase-3 activity (Guigon et al., 2009). Guigon et al. also analyzed the effect of a treatment with a specific mTORC1 inhibitor (RAD001) in the *TRβ*$^{PV/PV}$, *Pten*$^{+/-}$ mouse model. Although the treatment did not prevent capsular and vascular invasion and the occurrence of lung metastases, it considerably reduced thyroid tumor growth, there by prolonging the mice's life span. RAD001 induced a significant decrease in cell proliferation that was linked to the reduced amount and altered activity of key regulators of cell cycle progression (Guigon et al., 2010).

The steroid receptor activator-3 (SRC-3) is a member of the p160 family that complexes with members of the steroid/thyroid hormone receptor superfamily to modulate their transcriptional activity (Liao et al., 2002; Lonard and O'Malley, 2005). It is amplified in breast and ovarian cancer (Anzick et al., 1997) and overexpressed and/or amplified in other steroid hormone-sensitive tumors, such as prostate cancer and meningioma, as well as steroid hormone-independent tumors, such as pancreatic cancer, colorectal carcinoma, and hepatocellular carcinoma (Yan et al., 2006). Transgenic mice overexpressing *SRC-3* display many types of malignancy in several tissues, such as mammary gland, pituitary, uterus, lung, and liver, through activation of the insulin growth factor (IGF)/AKT signaling (Torres-Arzayus et al., 2004). To determine its effects on thyroid carcinogenesis, Ying et al. (2008) crossed *TRβ*$^{PV/PV}$ mice with *SRC-3* null mice to evaluate thyroid tumor development and progression. *TRβ*$^{PV/PV}$ mice deficient in SRC-3 (*TRβ*$^{PV/PV}$ *SRC-3*$^{-/-}$ mice) exhibited significantly increased survival, decreased thyroid tumor growth, delayed tumor progression, and lower incidence of distant metastasis as compared with *TRβ*$^{PV/PV}$, *SRC-3*$^{+/+}$ mice (Ying et al., 2008). Additional *in vivo* and *in vitro* analyses showed that SRC-3 deficiency leads to inhibition of cell cycle progression by controlling the expression of E2F1, could induce apoptosis through the control of the expression of *Bcl-2* and caspase-3, and finally could suppress neovascularization and metastasis (Ying et al., 2008).

PTEN$^{-/-}$ MODELS

Despite all the correlative data regarding the PI3K/AKT signaling cascade in the control of normal thyroid function and the link between its deregulation and thyroid disease, the aforementioned pathogenetic models still lacked direct *in vivo* evidence supporting their validity. To address these issues, we have generated a mouse strain in which the *Pten* gene is selectively deleted in the thyroid follicular cells by Cre-mediated recombination. This leads to constitutive activation of the PI3K/AKT pathway and reproduces the genetic events taking place in the nodular lesions and thyroid carcinomas developing in Cowden disease patients, who carry a heterozygous germline mutation in *PTEN*. *Pten* mutant mice develop from birth diffuse goiter characterized by extremely enlarged follicles, in the presence of normal TSH and T4 hormone levels (Yeager et al., 2007). A unique feature of this model is the differential proliferative advantage of mutant thyrocytes between genders, mimicking the increased incidence of thyroid disease in women (Yeager et al., 2007; Antico-Arciuch et al., 2010).

Surprisingly, goitrogen treatment did not cause a substantial increase of the mutant thyroid size and increased only to some extent the proliferation index of the female thyrocytes, suggesting that a relevant part of the TSH-induced proliferation signals are conducted through the PI3K/AKT cascade (Yeager et al., 2007), converging on mTOR (Yeager et al., 2008).

Complete loss of *Pten* leads to invasive tumors in over 50% of the mutant females and 35% of the mutant males aged 1 year (Antico-Arciuch et al., 2010). These data show that Akt activation is sufficient, *in vivo*, to induce thyroid hyperplasia and diffuse colloid goiter in young mice by increasing the thyroid mitotic index, and that it needs additional genetic alterations for neoplastic transformation to occur in older mutants.

Thyroid cancer originating from the follicular epithelium often displays activating mutations of Ras family members (Shi et al., 1991). Although several transgenic approaches have been used in the past to define the molecular mechanisms of Ras-mediated thyroid transformation, it is now clear that supraphysiologic expression in transgenic systems does not resemble the activity of oncogenic Ras when expressed at endogenous levels (Feunteun et al., 1997; Tuveson et al., 2004; Vitagliano et al., 2006).

The frequencies and overlap of genetic alterations in the PI3K and Ras/MAPK cascades increase with progression from differentiated to undifferentiated thyroid tumors: alterations in PI3K and MAPK pathways occur in nearly all undifferentiated tumors, with the majority of the cases harboring genetic alterations in both pathways (Hou et al., 2007).

To gain insights into how PI3K activation cooperates with activation of Ras in promoting thyroid cancer pathogenesis, the Kras mutant allele, G12D, was conditionally expressed in the thyroid epithelium through Cre-mediated deletion of a floxed STOP cassette preventing *KrasG12D* expression in mice that had already *Pten* deleted (Miller et al., 2009). Although each of these two pathways, alone, was unable to transform thyroid follicular cells, their simultaneous activation was highly oncogenic, leading to invasive, and metastatic follicular carcinomas (Miller et al., 2009). In particular, PI3K activation suppressed Kras-initiated feedback signals that uncouple MEK and ERK activation, thus inhibiting MAPK activity; in addition, PI3K and Kras cooperated to dramatically up-regulate cyclin D1 mRNA levels (Miller et al., 2009). Finally, combined pharmacologic inhibition of PI3K and MAPK completely abolished the growth of double-mutant transformed cell lines, providing a strong rationale for the dual targeting of these pathways in thyroid cancer (Miller et al., 2009).

Cell cycle progression is accomplished by a sequential and concerted activation of a family of serine–threonine kinases, named the cyclin-dependent kinases (CDKs). p27^{Kip1} is an essential CDK inhibitor and plays a fundamental role in key cellular processes such as proliferation, differentiation, apoptosis, substrate adhesion, and motility. Downregulation of p27^{Kip1} nuclear level or its cytosolic localization are always correlated with poor prognosis of numerous types of human epithelial and non-epithelial cancers (Borriello et al., 2011).

When analyzing the outcome of thyroid-specific *Pten*$^{-/-}$; *p27*$^{-/-}$ mice, the double-mutant animals exhibited a decrease of

their survival, and all died by 6 months of age, with dramatically hyperplastic thyroids that caused dyspnea and prevented feeding (Antico-Arciuch et al., 2010). Interestingly, *Pten*$^{-/-}$; *p27*$^{+/-}$ mutants had a mean survival of 58 weeks, a 25% reduction compared to *Pten*$^{-/-}$; *p27*$^{+/+}$ mice. While *Pten*$^{-/-}$; *p27*$^{+/+}$ females show a marked decrease in life span compared to single mutant males, these gender-based differences in survival were totally rescued by the reduction of *p27* gene dosage, suggesting that p27 is a relevant mediator of the effects of estrogen on thyroid tumor incidence (Antico-Arciuch et al., 2010). In fact, when we compared the proliferative index of thyroids from young, tumor-free *Pten*$^{-/-}$; *p27*$^{+/+}$ and *Pten*$^{-/-}$; *p27*$^{+/-}$ mice, we observed that loss of one *p27* allele in the males resulted in a larger proliferation increase than it did in the females. As a consequence, the gender differences in thyrocyte proliferation were considerably reduced in *Pten*$^{-/-}$ *p27*$^{+/-}$ mice. Furthermore, *Pten*$^{-/-}$; *p27*$^{+/-}$ compound mutants survived long enough to develop adenomas and carcinomas (Antico-Arciuch et al., 2010). These compelling data strongly support a role for p27 as an important mediator of estrogen signaling in thyroid hyperproliferation and neoplastic transformation.

CONCLUSION

Despite some shortcomings associated with a number of biological differences between mice and humans, the last 15 years have seen the development of a variety of genetically defined models of well differentiated thyroid tumors that have dramatically increased our knowledge of the key molecules and pathways involved in both neoplastic transformation and tumor progression. The challenge is now twofold: to extend these models to more aggressive tumor types (i.e., anaplastic carcinomas), in order to identify the specific mechanisms leading to loss of differentiation and increased metastatic potential; and to exploit these models and available cutting edge technology to pinpoint key molecular nodes that can be targeted in order to increase or restore therapeutic efficacy.

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