Lessons from Mouse Models of Thyroid Cancer

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Background: Thyroid cancer is the most common endocrine tumor and is increasing in incidence. The aim of this study was to review mouse models of differentiated thyroid cancer and how they elucidate human thyroid cancer biology.

Summary: Differentiated thyroid cancer, primarily papillary and follicular, comprises the majority of thyroid cancers. There has been tremendous growth in the cross-talk between basic science and clinical practice for thyroid cancer management. Insight into the framework of genes responsible for differentiated thyroid cancer has been gained through the use of mouse models. Common genetic alterations found in human papillary thyroid cancer such as *RET/PTC* rearrangements or the *BRAF*^{V600E} mutation have genetically modified mouse counterparts. These and other preclinical mouse models have validated the importance of the cyclic adenosine monophosphate (cAMP)/protein kinase A and mitogen-activated protein kinase (MAPK) signaling pathways in papillary thyroid cancer (PTC). *RAS* mutations have a role in both papillary and follicular thyroid cancer development. Mice with overactivation of the phosphatidylinositol-3-kinase (PI3K)–AKT and/or thyrotropin-regulated signaling pathways have been found to develop follicular thyroid cancer. Additional mouse models of thyroid cancer that utilize inducible expression systems are in development or are being characterized and will better reflect the majority of human thyroid cancers which are non-hereditary. Advances in *in vivo* imaging of mice allow for earlier detection of metastasis and the ability to follow tumor growth or regression which may be used in evaluation of pharmaceutical agents.

Conclusions: Mouse models have expanded our understanding of the altered signaling pathways that contribute to thyroid cancer tumorigenesis and provide a powerful tool to develop novel diagnostic approaches and therapies.

Introduction

THYROID CANCER is one of the few cancers whose incidence is rising and is the most common endocrine neoplasm (1). Differentiated thyroid cancers originate from the thyroid follicular cell, with the most common subtype papillary thyroid cancer (PTC) representing over 80% of thyroid malignancies and follicular thyroid cancer (FTC) and its variants comprising the second largest group (2). Other histologic groups of primary thyroid cancers include poorly differentiated and undifferentiated thyroid cancer, medullary thyroid cancer derived from the parafollicular C cells, and other rare primary thyroid cancers such as lymphoma (3). Although arising from the same epithelial cell, there are clear distinctions in tumor biology between PTC and FTC. Thyroid cancer patients typically have an excellent prognosis although patients with metastatic disease may have mortality rates approaching 50% over 10 years, but patients with FTC have a poorer outcome than PTC (4).

Standard therapeutic approach for differentiated thyroid cancer patients includes surgery and thyroid hormone suppressive therapy to reduce thyrotropin (TSH) levels, often followed by radioactive I-131 treatment, but few good options exist for metastatic disease (3). Recently, the class of targeted agents known as tyrosine kinase inhibitors (TKIs) has shown benefit for patients with advanced disease (5–8). The new American Thyroid Association (ATA) guidelines on thyroid cancer management state that TKIs may be considered for patients not in a clinical trial and with progressive or symptomatic metastatic disease (9). The burgeoning clinical interest in TKIs and other targeted therapies is partly due to the improved knowledge regarding the molecular basis of thyroid cancer.

There is a wide spectrum of scientific approaches available to model thyroid cancer and each has its own unique limitations. A major challenge of using human thyroid cancer tissue is its limited availability to compare genetic, epigenetic,

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and/or proteomic profiles between normal and malignant tissue along with patient heterogeneity. Cultured thyroid cancer cell lines are useful in dissecting out signaling pathways and predicting tumor response to pharmaceutical agents. However, the basic functional unit of the thyroid, the three-dimensional thyroid follicle, is lost in monolayered cultures. In two-dimensional culture systems, thyrocytes may also lose normal functions such as TSH responsiveness (10). Culture systems with three-dimensional matrices or other growth factors can now recreate thyroid follicles *in vitro* and provide additional information on tumor–stromal interaction (11–13). A more recent concern raised by Schweppe *et al.* is that many of the existing thyroid cell lines are redundant or are not thyroid in origin (14).

Mice are a fundamental tool for cancer investigators and provide insight into tumor behavior that cannot be replaced, at yet, by *in vitro* modeling. The mouse strain B6C3F1, frequently used in bioassay and carcinogenicity studies, has a very low incidence of spontaneous thyroid cancer development, which makes it difficult to incorporate in studies (15). There are three general approaches for inducing tumors: carcinogenic compounds, implantation of tumor cells via a subcutaneous or orthotopic route in immunocompromised mice, or genetically engineered models. Chemical compounds inducing thyroid cancer are typically goitrogens which decrease thyroid hormone levels, leading to TSH elevation and goiter development followed by infrequent development of follicular neoplasms (16). Immunodeficient mouse strains used to study cultured human cell lines *in vivo* include the severe combined immunodeficient (SCID) and athymic (nude) strains. There are subtle differences in their immune systems which may affect tumor development as SCID mice can also tolerate human lymphocytes unlike nude mice (17). The tumor implantation site influences tumor behavior as studies on thyroid and nonthyroid cancer cell lines show that growth and metastasis are facilitated if implanted orthotopically (18,19). As several published studies of orthotopic models of thyroid cancer used cell lines that are now found to be derived from colon cancer or melanoma (14), one must be careful in the conclusions previously drawn about thyroid cancer biology. Orthotopic models of thyroid cancer have been shown to develop lymph node and pulmonary metastases, but not other sites of distant metastasis (20). To model bone metastasis, intracardiac or direct injection of tumor cells into bone is required (21). One caveat to determining whether a distant metastasis is present is that it may be limited by the investigator's motivation and resources to look for unusual sites of distant metastasis such as the liver or brain. Similar to the techniques that are now clinically used to detect human thyroid cancer, advances in microimaging such as high-frequency ultrasound, microcomputed tomography, and micro-positron emission tomography will improve tumor detection in mice (22). Recent development of an orthotopic model of anaplastic thyroid cancer with a cell line that stably expresses green fluorescent protein showed the feasibility of detecting thyroid cancer metastasis in lymph nodes and lung by fluorescence imaging (23). Sequential visualization of cell lines that express luminescent proteins in *in vivo* studies can be used in the future to quantify tumor progression and eventual response to therapeutic agents (17).

Genetically engineered models include the traditional transgenic mouse which is created by injecting DNA, in-

cluding the gene of interest, into fertilized oocytes, resulting in random integration and unpredictable copy numbers of the foreign DNA into the mouse genome (24). Several transgenic mouse models of thyroid cancer contain the gene of interest under the expression of the thyroglobulin (Tg) promoter, allowing for thyroid-specific expression as shown in Table 1 (25–27). Manipulation of embryonic stem cells via homologous recombination allows for targeted gene manipulation and is the principle behind both knockout mice, which typically have no functional protein expression of the target gene, and knock-in mice, which have the transgene inserted at a specific locus. As all cells of these knockout and knock-in models contain the transgene, these are not representative of the majority of thyroid cancer cases that result from somatic mutations (28). In addition, transgenic and knock-in models have much earlier expression of the oncogene compared with acquired human thyroid cancer and these mice may develop compensatory mechanisms, limiting the opportunity to study thyroid cancer development and progression (25,29). To circumvent these problems, recombinase-mediated gene modification (*Cre/Lox*) is now used to introduce a somatic mutation in specific tissues and/or at a specific time (24,30–32). In one example of the *Cre/Lox* system, an exon of the gene of interest is modified by flanking it with two 34-bp *loxP* sequences (i.e., floxed). These mice are then crossed with a mouse strain that expresses the *Cre* recombinase, leading to deletion of the floxed sequence and gene inactivation (Fig. 1). Another model, described in more detail below, is the *Rap1b^{G12V}* model wherein a floxed, constitutively active mutant *Rap1b* gene upstream of an inactive *Rap1b* under control of the bovine Tg promoter is crossed with a tamoxifen-inducible *Cre* mouse so that in the presence of tamoxifen, *Rap1b* is inactivated (Fig. 1) (33). These advances in genetically engineered mice allow for both spatial and temporal control of gene expression.

Multiple variations of this approach have been designed for both oncogene activation and inactivation as exemplified in a lung cancer model (30). The sequence contains an oncogenic *K-Ras* mutation downstream of a floxed stop signal. Instead of crossing these mice with *Cre* mice, *Cre*-recombinase is delivered locally to the lungs via an adenovirus vector (30). When compared with the mouse model in which mutant *Ras* is expressed throughout the lung, this model recapitulates human lung cancer progression better, from a single individual somatic cell harboring an activated oncogene to macro disease (30).

Despite recent progresses, models generated from spontaneous mutations are rare (34). Another model that closely mimics sporadic somatic mutations uses endogenous intrachromosomal recombination mechanisms to simulate the random somatic activation of *K-Ras*. To make the chromosome susceptible for a mutagenic recombination event, two copies of exon 1 with one or two mutant alleles were inserted into the genomic site of the endogenous *K-Ras* allele in tandem. Intrachromosomal recombination between homologous sequences generates a single functional oncogenic *K-Ras* allele. In this model, all of these mice developed early onset lung adenocarcinomas (35).

The remainder of this review will focus on the genetically engineered models for differentiated thyroid cancer, specifically PTC and FTC. These models have enhanced our understanding of key signaling pathways central to thyroid carcinogenesis and can be used for future therapeutic agents.

TABLE 1. MOUSE MODELS OF PAPILLARY THYROID CANCER

Mouse line	Promoter/ expression	Strain	Pathology	Human correlates	Metastasis	Anaplasia	Thyroid function	Comments	References
<i>RET/PTC1</i> (Santoro <i>et al.</i>)	Rat Tg; thyroid	C57BL/6J	4/18 (22%) diagnosed with PTC starting at 8 months	PTC with <i>RET/PTC1</i>	No	No	NA	Three transgenic lines created, all with similar rate of PTC development	(26)
<i>RET/PTC1</i> (Jhiang <i>et al.</i>), high copy number	Bovine Tg; thyroid	FVB/N	100% mice with multifocal PTC; early tumor onset (earliest 4 days old); higher proliferation rate than low copy <i>RET/PTC1</i> mice	PTC with <i>RET/PTC1</i>	No	Rare	Congenital hypothyroidism	Decreased iodine uptake; administration of levothyroxine delayed PTC development and progression in both low and high copy mice	(25,32)
<i>RET/PTC1</i> (Jhiang <i>et al.</i>), low copy number	Bovine Tg; thyroid	FVB/N	100% mice with multifocal PTC; later tumor onset (earliest 21 days old).	PTC with <i>RET/PTC1</i>	No	No	Normal	All mice developed PTC; cross with <i>p53^{-/-}</i> mice resulted in liver metastasis, dedifferentiated tumors	(25,32)
<i>RET/PTC3</i>	Bovine Tg; thyroid	C3H/He	4/13 (31%) PTC by 3 months and 6/11 (55%) over 3 months; presence of solid-type PTC	PTC with <i>RET/PTC3</i> typically more aggressive than <i>RET/PTC1</i> , solid tumor variant of PTC and found in younger patients	Lymph nodes	No	NA	Three transgenic lines with similar <i>RET/PTC</i> expression; cross with <i>p53^{-/-}</i> mice resulted in aggressive, dedifferentiated tumors which grew in SCID mice	(49,50)
<i>BRAF^{V600E}</i> (Tg- <i>BRAF2</i>)	Bovine Tg; thyroid	FVB/N	14/15 (93%) PTC at 3 months; 13/14 (93%) 5+ months	Most common mutation in PTC; all types of PTC present, majority of tall-cell variant of PTC	Lymph nodes	Yes	TSH increased up to 80-fold; normal T4 and T3 levels	Higher levels of <i>BRAF^{V600E}</i> compared with Tg- <i>BRAF3</i> ; local invasion, dedifferentiation also seen	(27)

(Continued)

TABLE 1. (CONTINUED)

Mouse line	Promoter/ expression	Strain	Pathology	Human correlates	Metastasis	Anaplasia	Thyroid function	Comments	References
<i>BRAF</i> ^{V600E} (Tg- <i>BRAF</i> 3)	Bovine Tg; thyroid	FVB/N	3/12 (25%) PTC at 3 months; 4/9 (44%) at 5+ months	<i>BRAF</i> mutations more common in PTC that dedifferentiates	No	No	TSH increased up to twofold; normal T4 and T3 levels	No dedifferentiation seen	(27)
<i>TRK-T1</i>	Bovine Tg; thyroid	B6C3F1	6/26 (23%) < 7 months developed thyroid cancer (similar to classic PTC); 7/9 (78%) > 7 months developed PTC	Small % of radiation-induced PTC contain <i>TRK-T1</i> translocation	No	No	NA	Some mice developed a solid-type papillary thyroid cancer; <i>TRK-T1</i> crossed with <i>p27</i> ^{-/-} mice have increased incidence of PTC with shorter latency	(51,52)
<i>H-Ras</i> ^{G12V} (four transgenic mice M1-M4; two lines M3, M4)	Bovine Tg; thyroid	Mixed, mated with C57BL/ 6J.DBA/2J and DBA/2J	M1, M2, M4: PTC; M3: no cancer	Less frequent mutation in PTC	Unclear; M2, M4 lines with lung nodules	NA	Normal T4 level	Progeny from M4 developed lung cancer, but no thyroid cancer; No line was able to transmit PTC phenotype	(74)
<i>Ptkar1a</i> ^{Δ2/+}	Heterozygous knockout; ubiquitous	Mixed; > 50% FVB/N	4/44 (9%) solid- pattern thyroid cancer; papillary features	Carney complex	No	NA	NA	Earliest age of tumor onset was 13.5 months	(42)

RET, rearranged during transfection; PTC, papillary thyroid cancer; Tg, thyroglobulin; *TRK-T1*, neurotrophic tyrosine kinase receptor, type 1; TSH, thyrotropin.

FIG. 1. Examples of genetically engineered mice utilizing thyroid-specific promoters and *Cre* recombinase. **(A)** Thyroid-specific expression of an oncogene. The oncogenic target gene is placed downstream of a stop signal flanked by two *loxP* sites preventing its expression. Excision of the stop cassette with *Cre* recombinase allows target gene expression only in the thyroid. **(B)** Knockdown of a tumor suppressor gene by its excision through *Cre* recombinase. *Cre* is under control of a thyroid-specific promoter (like thyroglobulin) and its expression restricted to the thyroid, resulting in gene knockdown only in the thyroid. **(C)** Spatial and temporal gene expression by using a conditional inducible *Cre* recombinase. *Cre* is fused to the ligand binding domain of the estrogen receptor (ER) that can only bind to tamoxifen and not estrogen (ER-Tamoxifen). *Cre* is active only in the presence of tamoxifen and the target gene is only expressed in the thyroid due to its tissue-specific promoter.

PTC

The known genetic alterations found in PTC primarily affect two central signaling pathways in thyroid cells: TSH receptor (TSHR)-mediated signaling and mitogen-activated protein kinase (MAPK) pathways (Fig. 2). There is cross-talk between these pathways, and although mouse models to evaluate overactivation in either signaling pathways have been engineered, it is the models altering MAPK signaling which consistently recapitulate human PTC.

TSHR and cAMP signaling

The TSHR is a classical G protein-coupled receptor and TSH binding to TSHR leads to activation of the heterotrimeric G protein (36). Then the stimulatory subunit *G α* dissociates from the G protein complex with eventual activation of adenylyl cyclases and generation of the secondary messenger, cyclic adenosine monophosphate (cAMP), leading to protein kinase A (PKA) signaling (37) (Fig. 2). The importance of this mitogenic pathway's role in thyroid cancer is the basis for thyroid hormone suppressive therapy in patients. However, it is unclear as to the importance of the TSHR/cAMP/PKA pathway in thyroid cancer initiation. *G α* mutations are only found in a small percentage of thyroid cancer, and a minority of patients with McCune-Albright syndrome who have up-regulation of cAMP production develop thyroid cancer (38).

Mice made biochemically hypothyroid by antithyroid drugs have not reliably developed thyroid cancer despite dramatic TSH elevations. The adenosine A2 receptor also signals through G proteins and activation of cAMP/PKA signaling,

but transgenic mice expressing the canine adenosine A2 receptor developed goiters and hyperthyroidism, but no thyroid cancer (39). Two other models which mimic TSHR overactivation via constitutive activation of *G α* include the *Tg-gsp* mouse which has a constitutively active *G α* subunit under control of the Tg promoter and a mouse that had thyroid-specific cholera toxin A1 subunit expression (40,41). Despite development of goiters and hyperthyroidism, these models did not develop thyroid cancer. A mouse model of PKA overactivation which recapitulates many findings of the human syndrome of PKA overactivation, Carney complex, was generated using heterozygous mice for a null allele of *Prkar1a*, the type 1a regulatory subunit of PKA. Only about 9% of these mice developed thyroid cancers, primarily identified as PTC; however, the earliest age-of-onset was 13.5 months (42). It appears that in mice, TSHR and/or PKA overactivation is not sufficient to initiate tumorigenesis but does enhance existing progression of PTC. Transgenic mice expressing the human papillomavirus E7 protein developed both PTC and FTC although this protein is not known to be important in human thyroid cancer (43). When these transgenic mice were crossed with the mice expressing the adenosine A2 receptor, these tumors became more aggressive, including development of metastasis (44). Additional work is required to understand the differences in human and mouse response to TSH/cAMP/PKA overactivation and thyroid cancer development.

RET/PTC mutations

Chromosomal translocations involving the proto-oncogene rearranged during transfection (*RET*) is one of the earliest

FIG. 2. Altered signaling pathways and mouse models of thyroid cancer. Mouse models are indicated by an (*). Aberrant signaling through receptor tyrosine kinases (RTK) such as the *RET* and *NTRK1* receptors induce papillary thyroid cancer (PTC). Overactivation of the RAS/RAF/MEK/ERK pathway (or MAPK signaling pathway) leads to PTC. The *BRAF*^{V600E} mutation results in constitutive activation of BRAF and is commonly found in PTC. *RAS* overactivation is common to both papillary and follicular thyroid cancer (FTC) models. TSH receptor (TSHR) signaling via adenylyl cyclases/cAMP/protein kinase A (PKA) is important for normal thyroid growth, but only a few models of TSHR overactivation develop thyroid cancer (*Prkar1a*^{Δ2/+}, *Rap1b*^{G12V}). The adrenergic receptor may also contribute to thyroid cancer development also through increased cAMP and signaling via pathways such as MAPK and protein kinase C (PKC). Overactivation in the PI3K-AKT signaling pathway via cell-surface receptors such TSHR or inactivation of the phosphatase, *PTEN*, are important for FTC tumorigenesis. *TRβ*^{PV/PV} mice also have PI3K-AKT overactivation and develop FTC. Please see text for additional details regarding these mouse models.

identified genetic mutations in PTC (45). *RET* is normally not present in thyroid epithelial cells, but somatic *RET/PTC* mutations are commonly found in PTC, particularly radiation-induced thyroid cancer (37,46). *RET* is a tyrosine kinase receptor and chimeric *RET/PTC* proteins involve fusion of the 3' end of the *RET* gene with various 5' partner genes, referred to as PTC, leading to constitutive *RET* activity and increased signaling through various pathways, most notably the MAPK signaling cascade (46). Clinically, *RET/PTC1* (partner gene *H4*) PTC have a similar clinical course of adult-onset PTC, but *RET/PTC3* (partner gene *ELE1*) is associated with a more aggressive, solid variant of PTC.

Mouse models have consistently shown that *RET/PTC* rearrangements can initiate thyroid carcinogenesis. Two independent groups have generated thyroid-specific *RET/PTC1* transgenic mice (25,26) (Table 1). Santoro *et al.* generated mice expressing *RET/PTC1* under the control of the rat Tg promoter, but only about half developed PTC over a long latency

period and had no distant metastasis (26). Jhiang *et al.* utilized the bovine Tg promoter and generated two groups of *RET/PTC1* mice, low and high copy numbers of the transgene, with distinct tumor behavior (25,47). All of these *RET/PTC1* mice developed PTC; the low copy number mice had a longer latency time until development of PTC (up to 6 months), whereas the high copy number group had PTC as soon as several days following birth (47). The high copy line had congenital hypothyroidism and it is interesting to note that thyroid hormone replacement could delay tumor development and progression, suggesting the importance of TSH stimulation in this model. Metastasis was also absent in both mouse lines. In mice, *RET/PTC1* cancers requires additional mutations, such as knockout of the tumor-suppressor *p53*, to result in metastasis (48).

Transgenic *RET/PTC3* mice develop PTC, similar to the human solid variant of PTC, and about one-third develop axillary lymph node metastasis unlike their *RET/PTC1* mouse

counterparts (49). On comparing the genetic profiles by microarray analysis of these murine tumors to human PTC, the similarities were most congruent when using mouse PTC tumors at a young age (i.e., 2 months) (50). In particular, mouse and human PTCs had similar patterns of overexpression of genes involved in cell cycle, DNA repair, and genes associated with the immune response (50). As proof-of-concept that these mice are a model for human PTC, both also had similar patterns of expression for proteins involved in the MAPK pathway. As these *RET/PTC* mutations are germline in mice versus acquired in humans, PTC development and tumor progression may be more advanced in mice and therefore younger specimens may correlate better with human tumors. Nonetheless, *RET/PTC* mouse models have expanded our ability to understand the initiators of PTC.

NTRK1

A second chromosomal rearrangement involving the neurotrophic tyrosine kinase receptor, type 1 (*NTRK1*), has been found in PTC (37). *NTRK1* rearrangements (*TRK*) include diverse partner genes such as *TPM3*, *TPR*, and *TPG* known as -T1, -T2, and -T3 which result in constitutive activation of this receptor and overactivation of diverse pathways, including MAPK (51). These chromosomal rearrangements are less common in sporadic PTC than *RET/PTC*, but also suggest how ionizing radiation is detrimental to thyroid. The *TRK-T1* transgenic mouse model was important as *in vitro* *TRK-T1* cannot transform cells, and therefore, it was not clear as to its importance in thyroid cancer. About half of the transgenic mice that expressed *TRK-T1* developed thyroid cancer, either FTC or PTC, without distant metastasis (15). When crossed with knockout mice of the cell cycle inhibitor, p27, these double-mutant mice developed thyroid cancers at an earlier age (52). These studies show that *TRK-T1* is oncogenic and that loss of p27 may facilitate PTC development. Interestingly, *TRK-T1* mice have been used to evaluate whether sonographic features used for human thyroid nodules can predict malignancy in mice. Similar to humans, mouse thyroid nodules with at least two of the following features were classified as malignant: hypoechoic, microcalcifications, poor margins, irregular shape, and extension outside the thyroid gland (22).

BRAF^{V600E} and the MAPK pathway

The evolutionarily conserved MAPK signaling pathway is clearly important in PTC as evidenced by the *RET/PTC* mouse models. The MAPK pathway allows external signals to be transmitted to the cell through a signaling cascade leading to RAS activation, followed by RAF recruitment and activation which then activates the MAPK kinases (23) that phosphorylate and activate MAPK proteins (e.g., ERK) (Fig. 2). Dysregulation of MAPK signaling is common to many tumors, and mutations of *BRAF* are only second to *RAS* in terms of prevalence in tumors (53). The activating point mutation T1799A leads to a valine to glutamate substitution at position 600 of *BRAF* (*BRAF*^{V600E}) and is commonly found in PTC (54,55). *BRAF*^{V600E} is also important for prognosis as it is associated with the aggressive tall-cell variant of PTC and/or extrathyroidal extension and dedifferentiation (55).

To understand how *BRAF*^{V600E} affects PTC behavior, Knauf *et al.* created transgenic *BRAF*^{V600E} mice under control of the bovine Tg promoter (*Tg-BRAF*) (27). Two distinct lines,

Tg-BRAF2 and *Tg-BRAF3*, were studied, which differed in their level of *BRAF* expression. Interestingly, both lines had TSH elevation compared with wild-type mice, but the total thyroid hormone levels were the same. Goiters and PTC with similar histopathologic features of human *BRAF*-positive PTC occurred (Fig. 3). Higher expression of *BRAF* was associated with more aggressive PTC, including local invasion, but none of the mice developed distant metastasis. One clinical question that may be addressed by mouse models is how much TSH contributes to limiting aggressive thyroid cancer progression, such as tall cell. One issue with mouse models of thyroid cancer is that the Tg promoter is also regulated by TSH. Therefore, in this model and others, the higher TSH levels also led to increased oncogene expression which is not similar to human PTC (27). Recent work by Fagin and co-workers has created a mouse with doxycycline-inducible thyroid expression of *BRAF*^{V600E} (*Tg-rTta/TetO-BRAF*^{V600E}) which develops thyroid cancer in the presence of doxycycline and reversion of the malignant phenotype with removal of doxycycline and subsequent removal of *BRAF*^{V600E} (56). The overall shared features between mouse and human *BRAF*-positive tumors should provide additional insight for PTC.

FTC

FTC is a diagnostic challenge because of the inability to identify FTC on routine cytological examination by fine-needle aspiration biopsies of thyroid nodules. There has been significant progress in identifying genetic abnormalities in FTC, but none can be reliably used yet for diagnosis. Point mutations of *RAS* and rearrangement of the paired box 8 (*PAX8*)-peroxisome proliferator-activated receptor- γ (*PPAR* γ , *PAX8-PPAR* γ) are the most common genetic alterations. *PAX8-PPAR* γ rearrangement occurs in ~35% of FTC (57–59). Chromosomal t(2;3)(q13;p25) rearrangement results in the fusion of the thyroid transcription factor *PAX8* gene to the *PPAR* γ gene and overexpression of the *PPAR* γ protein (60). Tumors with *PAX8-PPAR* γ rearrangement tend to affect younger patients and frequently reveal vascular invasion (57,59). It is poorly understood on how the fusion protein causes FTC (61). A mouse model with the *PAX8-PPAR* γ under the control of the bovine Tg promoter does not develop FTC and only 25% develop hyperplasia (61,62). Another signaling pathway implicated in FTC is the phosphatidylinositol-3-kinase (PI3K)/AKT pathway, which is highlighted below and in Table 2. Other genetic alterations such as loss of *p53* function and β -catenin activating mutation have been detected late in tumor development (63–66).

RAS

RAS is a guanine nucleotide-binding protein that is a key intermediate in signal transduction pathways. *RAS* mutations are found in 40–50% of conventional follicular carcinomas and in 20–40% of adenomas (67–69). The most frequently affected hotspots are *NRAS* codon 61 and *HRAS* codon 61, although mutations of *RAS* involve codons 12, 13, and 61 of all three *HRAS*, *KRAS*, and *NRAS* genes (28,70). *RAS* activation depends on whether it binds to guanosine diphosphate (GDP) or guanosine triphosphate (GTP). It is inactive when it binds to GDP; when it binds to GTP, *RAS* initiates a downstream signaling pathway (71) (Fig. 2). The loss of the activity in

FIG. 3. *Tg-BRAF2* mice recapitulate papillary thyroid cancer (low-magnification, **A**), with overlapping, enlarged nuclei with nuclear grooves (**B**, arrow). Poorly differentiated carcinomas also develop (**C**) as well as tumors with aggressive features of tumor progression such as vascular invasion (**D**, arrow). Adapted from Figure 4 of Ref. (27). This figure was kindly provided by Drs. Jeffrey A. Knauf and James A. Fagin.

the conversion of GTP to GDP by *RAS* mutants results in constitutive activation of signaling in this pathway (71). The presence of a *RAS* mutation has been found to correlate with tumor dedifferentiation and a less favorable prognosis (72). The aggressive biological properties of these tumors may be due to the effect of the mutant *RAS* protein on promoting chromosomal instability, which has been demonstrated *in vitro* (73).

Transgenic mice expressing a mutated and activated *H-Ras*^{G12V} controlled by the bovine Tg promoter developed PTC with concurrent lung nodules; however, these lung lesions had a papillary architecture but more likely represented primary versus metastatic tumors (74). Although different founder strains were generated, with varying levels of *RAS* expression, these mice were limited in that they could not pass this phenotype to their progeny. An earlier mouse model containing a mutant *K-Ras*^{G12V} gene under the control of rat Tg promoter showed no thyroid cancer with normal thyroid hormone levels. Following treatment of these mice with goitrogens for 6 months, only one mouse developed FTC (75) (Table 2). This suggests that *RAS* mutation alone is not sufficient to induce FTC but acts as a predisposing factor and that multiple genetic alterations are required for FTC (75). *K-Ras*^{G12V} mice could be used for the study of genetic factors that might promote the development of FTC.

Another mouse model was created by targeting a human *NRAS* with a mutation at codon 61 (*N-Ras*^{Gln61Lys}) to thyroid follicular cells (76). Comparison of thyroid histopathology between these transgenic mice and wild-type littermates up to

18 months of age indicated progressive changes from hyperplasia to adenoma and carcinoma in some transgenic mice. Among 88 mice examined, 26 (30%) developed carcinomas that were follicular or had mixed papillary–follicular features and 9 (10%) had invasive carcinomas with large poorly differentiated areas closely resembling those observed in human patients. Distant metastases were observed in the liver of three mice, lung of two mice, and the right femur of one mouse (76). Although occurrence of metastasis requires long latency, the mice are potentially useful for studies of thyroid carcinogenesis.

A concern regarding these transgenic mouse models of FTC is that the cancer phenotype resulting from overexpression of a mutated *RAS* gene might not reflect the activity of endogenous mutant *RAS* expressed at the normal physiological level. It is not clear among these models whether the differences resulted from different functions of mutant genes, different expression levels, or other factors. Accordingly, a mouse model was created to express *K-Ras*^{G12D} under control of the endogenous *KRAS* promoter (34). Similar to other models, constitutive activation of *K-Ras*^{G12D} did not cause any physiologic or functional thyroid alterations. As aberrant activation of the PI3K pathway has been identified in most thyroid cancers (77,78), double-mutant mice with knock-in mutated *K-Ras*^{G12D} gene with *Pten*^{-/-} deletion targeted to the thyroid were created to examine whether mutant *KRAS* requires simultaneous PI3K activation to fully realize the cells' oncogenic potential. The concurrent activation of *K-Ras*^{G12D} and PI3K in thyroid follicular cells led to aggressive, invasive, and

TABLE 2. MOUSE MODELS OF FOLLICULAR THYROID CANCER

Mouse line	Promoter/ expression	Strain	Pathology	Human correlates	Metastasis	Anaplasia	Thyroid function	Comments	References
<i>K-Ras^{G12V}</i>	Rat Tg; thyroid	C57BL/6J, DBA/2	1/51 (2%) developed FTC only following goitrogen for 6 months, followed by suspension for 2 months	KRAS mutations are less prevalent than other RAS mutations in FTC	No	No	Normal (without goitrogen)	Other mice with disorganized follicle architecture	(75)
<i>N-Ras^{Gln61Lys}</i>	Bovine Tg; thyroid	Founders crossed with C67BL/6J	10/88 (11%) adenoma; 26/88 (29.5%) FTC/mixed follicular variant PTC; 9/88 (10%) poorly differentiated carcinoma	NRAS mutations may be associated with dedifferentiation	Liver, lung, bone	Poorly differentiated without typical features of anaplastic cancer	Elevated TSH (2.5- fold) in 60% mice 4-8 months; normal T4	30/35 (86%) developed thyroid cancers (FTC and poorly differentiated) after 18 months and 25% mice with cancer had dedifferentiation; <i>in vitro</i> <i>NRAS^{Gln61Lys}</i> increased genomic instability of PC C13 cells	(76)
<i>K-Ras^{G12D}</i> <i>Pten^{-/-}</i>	<i>K-Ras^{LSL}</i> , <i>TPO-Cre</i> ; <i>Pten^{L/L}</i> ; <i>Thyroperoxidase-Cre</i> (<i>TPO-Cre</i>); using human <i>TPO-Cre</i> , conditional thyroidal expression of <i>K-Ras^{G12D}</i> and loss of <i>PTEN</i>	129SV	100% FTC with local invasion; none survived over 4 months	FTC; model of cancer progression through accumulation of genetic mutations	Lung	NA	Low TSH, increased T4 (2-3-fold) suggests primary hyperthyroidism due to autonomy expression	<i>K-Ras^{G12D}</i> expression or <i>Pten</i> knockout alone did not cause thyroid cancers; knock-in of <i>K-Ras^{G12D}</i> results in its physiologic expression	(34)
<i>TRβ^{PV/PV}</i>	Knock-in; ubiquitous	C57BL/ 6J × 129SV	100% FTC; tumor is seen at 4-5 months; lung metastasis at 5+ months	Resistance to thyroid hormone syndrome; FTC	Lung	Yes	Increased TSH (>200- fold); increased T4 and T3 levels (7-8-fold)	Heterozygous mice do not develop cancer, but mice with one mutated <i>TRβ</i> and lacking wild-type <i>TRβ</i> develop cancer (<i>TRβ^{PV/-}</i>)	(79,83,95)
<i>α1b-adrenergic receptor</i>	Bovine Tg; thyroid	Founders crossed with C57BL/6J	six lines, three lines (51, 57, 56) with visible goiter and cancer (follicular, papillary, trabecular, solid)	Constitutive TSH signaling	Lung	NA	T4 varies by mouse line and age, ~2-5-fold elevation	20% metastasis in mice > 12 months	(103)
<i>Rap1b^{G12V}</i>	Bovine Tg; thyroid <i>Rap1b^{G12V}-LoxP-</i> <i>N17</i> ; <i>Cre-ER-Tx</i> ; conditional estrogen receptor inactivation with tamoxifen (ER-Tx)	FVB (Taconic)	Mice treated with a goitrogen for 12 months developed FTC with local invasion into muscle	TSH/cAMP overactivation	None	No	Normal (without goitrogen)	Inactivation of <i>Rap1b^{G12V}</i> reduced goiter size despite ongoing treatment with goitrogen	(105)

FTC, follicular thyroid cancer; cAMP, cyclic adenosine monophosphate.

metastatic follicular carcinomas (34). Fifty percentage of the mice died within 7 weeks after birth, and none survived longer than 4 months. Serum TSH level was drastically reduced and T4 was increased in these mice, suggesting thyroid autonomy. All mice surviving longer than 12 weeks developed lung metastases (34). The *K-Ras*^{G12D} mouse model would be useful to study the interaction of different pathways necessary for the development of metastatic thyroid cancer.

TRβ^{PV}

The *TRβ*^{PV} knock-in mouse was initially created to model an inheritable disease called resistance to thyroid hormone (RTH) (79). The hallmark of RTH is elevated serum thyroid hormone levels associated with nonsuppressible TSH (80,81). RTH is due to the mutations of the thyroid hormone receptor β (*TRβ*) gene. Most RTH patients are heterozygous with a mutated *TRβ* allele. Although the clinical symptoms of RTH are generally mild, one patient with a homozygous mutation died at a young age with extraordinarily high levels of TSH (80,81). *TRβ*^{PV} mice were created through homologous recombination by targeting a PV mutation to the *TRβ* gene (79). The PV mutation was identified in a patient with RTH (82) who has a frameshift mutation in the C-terminal 14 amino acids of *TRβ*, resulting in a complete loss of T3 binding and transcriptional capacity. *TRβ*^{PV} mice faithfully recapitulate human RTH (79).

Remarkably, as homozygous *TRβ*^{PV} (*TRβ*^{PV/PV}) mice age, they spontaneously develop FTC with a pathological progression similar to human FTC. As early as 2 months of age, *TRβ*^{PV/PV} mice exhibit papillary hyperplasia. By 4–5 months

of age, signs of FTC such as capsular and vascular invasion are already apparent. Distant metastases to the lungs and heart are common in mice older than 5 months (Fig. 4). The pathological changes progress from hyperplasia (100% of all mice examined), to capsular invasion (91%), vascular invasion (74%), anaplasia (35%), and/or distant metastasis (30%). Metastasis involves mainly the lung and occasionally the endocardium, but not local lymph nodes. The small, round, dark nuclei of the developed thyroid carcinomas are characteristic of follicular carcinoma (83). The *TRβ*^{PV/PV} mouse model has facilitated the identification of altered signaling pathways underlying FTC. Indeed, similar to human FTC, overactivation of tumor promoters [e.g., the pituitary transforming tumor gene, cyclin D1, and β -catenin (84–94)] and tumor-promoting signaling pathways (e.g., PI3K-AKT signaling) has been uncovered. The *TRβ*^{PV/PV} mouse serves as a preclinical model of FTC, having been used to test several potential therapeutic targets (85,92).

Spontaneous development of FTC has also been observed in mice with one mutated *TRβ*^{PV} but without the wild-type *TRβ* allele (*TRβ*^{PV/-} mice) (95). This *TRβ*^{PV/-} mouse was generated by cross-breeding *TRβ*^{PV/+} mice with *TRβ* knock-out mice (95). The pathological progression in the thyroid of *TRβ*^{PV/-} mice was indistinguishable from that of *TRβ*^{PV/PV} mice. These results indicate that in the absence of a wild-type allele, the mutation of one *TRβ* allele is sufficient for the mutant mice to spontaneously develop FTC. These results suggest that the *TRβ* could function as a tumor suppressor. Importantly, these findings raise the possibility that *TRβ* could be tested as a novel therapeutic target in thyroid cancer.

FIG. 4. *TRβ*^{PV/PV} mice recapitulate follicular thyroid cancer with capsular and vascular invasion (panels A and B respectively, arrows). Dedifferentiation can be found (panel C) along with distant metastasis to lung (panel D, arrow). Reproduced from Ref. (83). This figure was kindly provided by Dr. Sheue-Yann Cheng.

The $TR\beta^{PV/PV}$ mouse is a unique model, as it closely resembles human FTC in pathological progression and its underlying genetic alterations (84–94,96,97). Though it is not known whether the only reported patient with homozygous mutation of the $TR\beta$ gene (81) developed thyroid cancer, abnormal expression or somatic mutations of TR are associated with several human cancers including PTCs (98,99). The silencing of the $TR\beta$ gene by hypermethylation and the concurrent reduction of $TR\beta 1$ transcripts were demonstrated in breast cancer (100). In cotransfection experiments, both $TR\alpha 1$ and $TR\beta 1$ strongly repressed $HRAS^{G12V}$ -induced transformation of NIH3T3 fibroblasts, reduced tumor volume, and inhibited tumor growth in nude mice (101). Also, the transfection of $TR\beta 1$ into hepatocarcinoma and breast cancer cells reduced tumor growth, caused partial mesenchymal-to-epithelial cell transition, and had a striking inhibitory effect on invasiveness, extravasation, and metastasis formation in mice (102). The results from the $TR\beta^{PV/PV}$ mouse model are consistent with these reports that the $TR\beta$ receptors could act as a tumor suppressor in the development of FTC.

Mutant α_{1B} -adrenergic receptor

A mutated α_{1B} -adrenergic receptor gene under control of the bovine Tg promoter resulted in constitutive activation of the TSH signaling pathway and was used to establish a model for FTC (103). In the first few weeks of life, the mice began to develop goiters and by 12 months some thyroids were 130 mg which is over 20 times normal. As the mice aged, thyroid nodules developed, and in a few cases, all morphological criteria of thyroid follicular cell differentiation were lost; spindle-cell foci were found without formation of identifiable follicles. The foci frequently reached malignancy criteria for human thyroid tumors with local invasion into the muscle. Lung metastases were observed at an overall rate estimated to be 20% in mice older than 12 months. The manifestation of advanced tumor features in this mouse model may be useful for studies of the invasion and metastasis of FTC mediated by TSH-activated signaling pathways.

$Rap1b^{G12V}$

$RAP1$ is a member of the RAS family of small G proteins that transmit signals from the TSH receptor to the MAPK pathway by means of a cAMP-dependent mechanism in thyroid cells (104). $RAP1$ was shown to have both mitogenic and tumorigenic properties (105). In a mouse model, a thyroid-specific constitutively active $Rap1b$ ($Rap1b^{G12V}$) also allowed the inducible switch to the inhibitory form of $Rap1b$ ($Rap1b^{S17N}$) (33). Mice expressing $Rap1b^{G12V}$ showed no signs of hypothyroidism or hyperthyroidism. Exposure of $Rap1b^{G12V}$ mice to a 6-month goitrogen administration led to nodular thyroid enlargement which reversed after goitrogen removal. When expression of the $Rap1b$ gene was switched to the inactive $Rap1b^{S17N}$, the thyroid gland size decreased by 50% despite continued goitrogen treatment, because of decreased proliferation versus increased apoptosis. However, after long-term goitrogenic treatment for 12 months, some $Rap1b^{G12V}$, but no wild-type mice, developed signs of FTC without metastasis. $Rap1b^{G12V}$ appears oncogenic in the presence of elevated TSH-mediated signaling. The $Rap1b^{G12V}$ mouse model might be useful in studies of multiple factors that could contribute to the development of thyroid cancer.

Conclusions

The development of these mouse models of thyroid cancer have not only confirmed the transforming ability of single-gene alterations, such as RET/PTC translocations or $B-RAF^{V600E}$, in initiating thyroid cancer, but have also shown new genes responsible for FTC, such as $TR\beta$, and the importance of understanding additive and synergistic gene effects on thyroid cancer, such as $PTEN$ deletion and $KRAS$ over-activation.

Clinically, the presence of distant metastasis is the most important factor in determining a patient's prognosis. It is interesting to note that the models of PTC do not develop distant metastasis unless another mutation is introduced, whereas in FTC, three different mouse models of thyroid cancer with lung metastasis exist: $TR\beta^{PV/PV}$, a mouse model with simultaneous activation of RAS/PI3K pathway, and a mouse model of the mutant α_{1B} -adrenergic receptor (34,83,103). This may parallel human thyroid cancer, as FTC is more likely to have distant metastasis than PTC, or suggest a common pathway required for thyroid cancer metastasis. All three FTC models with metastasis share the features of an enlarged thyroid and an elevated TSH-mediated signaling pathway. In mouse models with RAS mutations, elevated TSH levels often promote the development of FTC. However, the precise role of TSH in thyroid carcinogenesis and whether it plays a more critical role in FTC tumorigenesis versus PTC remain to be fully elucidated.

Mouse models of thyroid cancer are important for developing therapeutic interventions. The roles of several signaling pathways, such as RAS-MAPK, and PI3K-AKT, in thyroid cancer are better understood by using transgenic mouse models. With the generation of mouse models of thyroid cancer, pharmaceutical agents may be developed and evaluated *in vivo* and on primary cell cultures from these mouse tumors prior to use in clinical trials. One of the limitations of many clinical trials for thyroid cancer is the inability to compare tumors pre- and posttreatment, to understand how targeted agents such as TKIs alter tumor phenotype. Coupled with radiologic imaging, mouse thyroid cancers could potentially be followed for response to a targeted agent, and tissue could be obtained before and after treatment for analysis. These preclinical models of thyroid cancer have opened many new and exciting research avenues which may have direct benefit to thyroid cancer patients in the near future.

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