HHS Public Access

Author manuscript

Mol Cell Endocrinol. Author manuscript; available in PMC 2017 February 05.

Published in final edited form as:

Mol Cell Endocrinol. 2016 February 5; 421: 18–27. doi:10.1016/j.mce.2015.06.029.

Mouse Models of Thyroid Cancer: A 2015 Update

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Abstract

Thyroid cancer is the most common endocrine neoplasm, and its rate is rising at an alarming pace. Thus, there is a compelling need to develop *in vivo* models which will not only enable the confirmation of the oncogenic potential of driver genes, but also point the way towards the development of new therapeutics. Over the past 20 years, techniques for the generation of mouse models of human diseases have progressed substantially, accompanied by parallel advances in the genetics and genomics of human tumors. This convergence has enabled the development of mouse lines carrying mutations in the genes that cause thyroid cancers of all subtypes, including differentiated papillary and follicular thyroid cancers, poorly differentiated/anaplastic cancers, and medullary thyroid cancers. In this review, we will discuss the state of the art of mouse modeling of thyroid cancer, with the eventual goal of providing insight into tumor biology and treatment.

Keywords

Thyroid Cancer; Mouse modeling; Genetics; Pre-clinical models

2. INTRODUCTION

Thyroid carcinoma is the most common endocrine malignancy. It affects about 1% of the population (Nikiforova and Nikiforov, 2008) and its incidence is on the rise across the globe and in United States, particularly in women (Jemal, Siegel, Ward et al., 2008). Although modern imaging techniques including ultrasonography, CT scanning, and nuclear imaging provide significantly enhanced detection of thyroid lesions, the rise in incidence appears to be greater than would be predicted solely from improved case finding (Enewold, Zhu, Ron et al., 2009).

The thyroid itself is composed of epithelial cells (thyrocytes), which form the thyroid follicles and secrete thyroid hormone, and C-cells, neuroendocrine-derived cells which

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inhabit the interfollicular spaces and secrete calcitonin. Cancers can arise from either cell type, although epithelial thyroid cancers make up 95–97% of human thyroid cancer. Epithelial thyroid cancers (also referred to as non-medullary thyroid cancers, NMTC) are divided into three types based on histology: papillary, follicular, and anaplastic. Of these subtypes, papillary thyroid cancer (PTC) is the most common, comprising about 80% of all thyroid cancers. Follicular thyroid cancer (FTC) accounts for another 10–15%, and anaplastic thyroid cancer (ATC) makes up to about 5%. The remaining few percent are medullary thyroid cancers (MTC).

Although the overall survival of patients with localized thyroid cancer is >95% at 5 years, there is a subset of patients who develop metastatic disease. The prognosis of these patients with metastatic thyroid cancer of any histopathologic subtype drops significantly, as thyroid cancers respond poorly to systemic therapy. By historical measures, the use of RAI can be considered the first example of 'targeted' therapy, but patients that are not cured by initial surgery followed by an appropriate RAI dose typically are not cured by subsequent doses. In the new era of targeted therapies, there has been significant excitement about the development of tyrosine kinase inhibitors targeted to the molecular abnormalities in thyroid (and other) cancer subtypes. In practice, these treatments have provided significant benefit, but the time of response tends to be self-limited. Thus, there is a pressing need for the development of new therapies which will be effective in the patient arena.

In the past, much of the work on new drug development relied on the use of cell line models, and there are well-established human cell line models for each of the major thyroid cancer subtypes. Drug studies can be performed *in vitro* in the tissue culture lab, or can be performed *in vivo* using xenografts into immunosuppressed mice. Traditional techniques have used subcutaneous tumor implants (Kim, Park, Schiff et al., 2005), although recent efforts to develop more physiologic systems have described the use of eutopic implants of thyroid cancer cells into the thyroid bed (Kim et al., 2005, Nucera, Nehs, Mekel et al., 2009). As a model of metastasis, thyroid cancer cells have also been injected into the tail vein or into the cardiac vasculature to produce widespread tumors (Li, Reeb, Sewell et al., 2013, Zhang, Gaskins, Yu et al., 2014). However, the limited effectiveness of agents which work well in these models points to the need to develop new therapies based on tumors which arise *in situ* in the immune competent host. To this end, there has been substantial interest over the past few years to develop genetically engineered mouse models for thyroid cancer. Not only do these models provide an *in vivo* confirmation of the genetic drivers of thyroid cancer, but they also provide an optimal setting for pre-clinical testing of new drug treatment paradigms. In the age of personalized medicine, it has been possible to generate mouse lines with the most common human mutations driving thyroid cancer. If a drug can be effective at treating a cancer in such a model system, there would then be a much stronger expectation that the drug would be effective in the corresponding patients, an approach supported by early data (Chakravarty, Santos, Ryder et al., 2011, Ho, Grewal, Leboeuf et al., 2013).

In this review, we will discuss the current state-of-the-art in the development of genetically engineered mouse models for human thyroid cancer. While traditional mouse modeling has relied on the production of transgenic lines by pro-nuclear DNA injection into blastocysts

(Hanahan, Wagner and Palmiter, 2007) and of knockout lines (either conventional or conditional) by homologous recombination in mouse embryonic stem (ES) cells (Capecchi, 1989), newer technologies have expanded the repertoire of possible models. The techniques include the introduction of cre-inducible alleles (using a so-called lox-STOP-lox cassette) (Soriano, 1999), the use of inducible exogenous genes (generally using a tetracycline/doxycycline inducible or suppressible system) (Schonig, Bujard and Gossen, 2010) and drug-inducible cre activity (generally the Cre-ERT2 transgene, which is activated by tamoxifen treatment) (Indra, Warot, Brocard et al., 1999). In addition, the capabilities of genome editing techniques such as TALENs (Hermann, Cermak, Voytas et al., 2014), zinc-finger nucleases (Sung, Baek, Seong et al., 2012), and the CRISPR/Cas9 system (Wang, Yang, Shivalila et al., 2013) are only beginning to be explored. Thus, although mouse modeling has reached a certain level of sophistication, the field should continue to evolve rapidly.

For now, a current survey of mouse models for NMTC resulting from single gene mutations and multi-gene mutations are presented in Tables 1 and 2, respectively, and models for MTC are presented in Table 3. The details of these models are discussed in the text. However, it is worthwhile recalling that once alleles are generated, they can be crossed to other mice in order to study tumor promotion or suppression. The sheer number of potential crosses makes fully cataloging all published crosses an endeavor which would only serve to cloud the value of the information presented. Thus, although we have tried to describe the major thyroid cancer models, this review is not meant to be an exhaustive list of all mice which have thyroid cancer as part of their phenotype. The key consideration is that as these models and the tools with which to analyze them become more sophisticated, it is expected that their value as pre-clinical models for therapeutic evaluation will continue to grow. Eventually, these mice should enable the development of new therapies which will improve our ability to treat patients with aggressive forms of thyroid cancer.

3. MOUSE MODELS OF PAPILLARY THYROID CANCER

3.1 Phenotype of human PTC

Papillary thyroid cancer (PTCs) are typically unencapsulated tumors characterized in humans by papillary architecture and a specific nuclear feature called “nuclear grooves”, which are easily noted by experienced thyroid pathologists. These tumors frequently exhibit overlapping nuclei with ground-glass appearance and invaginations of cytoplasm into the nuclei (Schlumberger, 1998). PTC exhibits histopathologic heterogeneity, and includes classic PTC (cPTC), follicular variant PTC (fvPTC), tall cell variant PTC (tcvPTC), and Hurthle cell carcinoma (HC). When metastatic, PTC typically spreads through lymphatic channels to lymph nodes, leading to involvement of neck nodes and then typically the lungs, although metastatic disease can also be observed in the liver, bones, or brain.

3.2 Molecular pathways and the Genetics of human PTC

The signaling hallmark of PTC is activation of the ERK signaling pathway, although levels of activation vary somewhat depending on tumor histology. ERK is the downstream effector

for membrane signaling through specific tyrosine kinase receptors, which in turn activate the RAS-RAF-MEK-ERK signaling cascade.

Of the components of this pathway, it has become clear over the past few years that the $BRAF^{V600E}$ mutation, which causes constitutive activation of the BRAF kinase, is the most common mutation driving sporadic PTC. This mutation is found in upwards of 40% of PTCs, and the most recent data from the cancer genome atlas (TCGA) suggests the number may be greater than 60%, at least in well-differentiated PTCs (Cancer Genome Atlas Research, 2014). The incidence of the $BRAF^{V600E}$ mutation varies by histotype, with a predominance of cPTC (60%) and tcvPTC (80%) but only 10% of fvPTC (Nikiforov, 2011).

Early studies of the genetics of PTC identified the presence of translocations of the RET tyrosine kinase, such that the C-terminal kinase domain was fused to another protein providing an N-terminal dimerization domain. RET-PTC gene fusions are thought to be responsible for 15–20% of PTC, and most of these arise in individuals with a history of radiation exposure (Nikiforova and Nikiforov, 2009). There are at least 11 fusion genes of RET which have been described (Santoro, Melillo and Fusco, 2006), the most common of which are designated as RET-PTC1 and RET-PTC3. Many of these fusions represent intrachromosomal rearrangements involving chromosome 10, where the *RET* gene is located (Santoro et al., 2006).

In RET-PTC1 fusions, the RET N-terminal kinase domain is fused to the 5′ end of the H4 gene, whereas RET-PTC3 is a fusion between the RET kinase domain and the *ELE1* (*NCOA4)* 5′ end. In these (and other) RET fusion proteins associated with thyroid cancer, the N-terminal domain of the RET fusion partner promotes dimerization and transphosphorylation, which appears to be required for cellular transformation (Fusco, Grieco, Santoro et al., 1987, Tong, Xing and Jhiang, 1997). Dissection of the activation of the RET kinase domain has demonstrated that the tyrosine 1062 residue is responsible for activating RAS/ERK, p38MAPK, JNK and PI3K/Akt pathways (Ichihara, Murakumo and Takahashi, 2004). Tyrosine 1062 is also important for activation of CREB and NF-κB transcription factors through RAS/ERK and PI3K pathways (Hayashi, Ichihara, Iwashita et al., 2000).

Similar to these observations with RET fusion proteins, the TRK family of oncogenes results from the fusion of carboxy terminus of NTRK1 with amino terminus of partner genes (Rabes, Demidchik, Sidorow et al., 2000). TRK fusions have been reported to occur with Chromosome 1 inversions which lead to the fusion of the TRK kinase domain with either the Tropomyosin gene *TPM3 or the TPR* gene (TRK-T1, T2, and T4) as well as with the *TFG* gene on chromosom*e 3.* NTRK fusions are very rare compared to the incidence of RET-PTC fusion oncogenes, although they also couple to the RAS-RAF-MEK-ERK signaling pathway.

Between receptor tyrosine kinases and BRAF lies the RAS family of oncogenes, which are well-known for their role in promoting multiple types of cancer. In the thyroid, RAS oncogenes are thought to account for ~10% of cases of PTC, with N-RAS most common (8.5%), then H-RAS (3.5%), and K-RAS (1%). These mutations tend to be observed in

patients with fvPTC. High throughput sequencing analysis done as part of the TCGA project has also suggested other potential drivers of PTC, including *EIF1AX, PPM1D, and CHEK2;* however, these have not yet been experimentally tested in thyroid models (Cancer Genome Atlas Research, 2014).

3.3 MOUSE MODELS OF PTC

3.3.1 BRAF tumor models—As described above $BRAF^{V600E}$ mutations account for majority of PTC cases and are also a sign of poor prognosis due to aggressive behavior of the tumors (Xing, Alzahrani, Carson et al., 2013) (Xing et al., 2013). There are currently multiple mouse models of BRAF activation, and the overall picture is highly consistent across models. The most straightforward model is a transgenic line targeting of $BRAF^{V600E}$ to the thyroid by the use of the bovine thyroglobulin (Tg) promoter (Knauf, Ma, Smith et al., 2005). Two different lines were generated using this strategy, differing in the level of overexpression of the transgene. In one line, thyroid cancer was highly penetrant, with PTC detected in >90% of animals by 12 weeks. The other line was similar, but with a reduced incidence of neoplasia (25% at 12 weeks). PTCs arising in these animals were most commonly the tall cell variant of PTC, and the more aggressive variant (the Tg-BRAF2 line) exhibited a 30% decrease in survival at 5 months. Cancers were locally invasive, although distant metastasis was not observed. It was felt in the majority of animals that death was due to compression of structures in the neck (i.e. esophagus and trachea). Two factors complicate the analysis of the TG-BRAF mouse lines. First, expression of the BRAF transgene led to dedifferentiation of cells, such that TG-BRAF mice exhibited significant elevation of TSH levels, likely as a result of ineffective production of hormones from the glands. Second, as the cells de-differentiated, they tended to lose expression of the Tg-driven transgene.

To overcome these issues, a second model was derived in which a thyroid-targeted $BRAF^{V600E}$ allele was driven by the tetO transactivator protein, which was exclusively expressed in the thyroid under the control of the Tg promoter (Tg-rtTA/tetO- $BRAF^{V600E}$ mice) (Chakravarty et al., 2011). This model enables expression of oncogenic BRAF in response to tetracycline (or doxycycline) treatment; notably, transgene expression ceases when the drug is withdrawn from the mice. $BRAF^{V600E}$ expression led to MEK and ERK activation and to tumor formation (8 fold increase in thyroid mass) after just one week of doxycycline exposure. The thyroid mass regressed significantly within 72 hours of doxycycline withdrawal, reaching normal size 1 week later. The histological features resembled solid growth pattern with nuclear features characterizing PTC such as nuclear grooves, enlargement of nucleus, overcrowding, overlapping and irregularity of nuclear contours. Interestingly, no metastatic lesions were observed. As in the constitutive model (Knauf et al., 2005), one week of doxycycline treatment led to global downregulation of thyroid-specific gene expression suggesting BRAF activation interferes with key regulatory factors important for their transcription. As an alternative approach to the same issue, mice were generated in which Tpo-driven cre was used to activate BRAF. Mice generated with this approach were runted and did not survive long, likely due to profound hypothyroidism and large goiters (Charles, Iezza, Amendola et al., 2011, Franco, Malaguarnera, Refetoff et al., 2011). To circumvent this issue, the group of McMahon developed a tamoxifen-

inducible cre allele (Cre-ERT2), which were then crossed to mice with a cre-activatable $BRAF^{V600E}$ allele (Charles et al., 2011). Mice were treated with Tamoxifen at 1 month of age, and followed for 12 months, during which time 100% of animals developed features of PTC. As in the other models, these mice exhibited loss of thyroid-specific differentiated functions caused by expression of the oncogenic BRAF transgene.

As with human PTC, these mice exhibit strong activation of ERK signaling. In order to test the viability of these models as potential tools for development of therapeutic strategies, mice were treated with agents in development, including the MEK inhibitor PD325901, which demonstrated reduction in tumor size as well as increases in tumor radioiodine uptake (Chakravarty et al., 2011, Charles et al., 2011). These studies eventually led to a similar trial in human patients, where the MEK inhibitor selumetinib caused increased therapeutic efficiency of RAI in patients (Ho et al., 2013). This model has also been used to assess the role of the microenvironment, where targeting of macrophages to the tumor reduced tumor growth in this mouse model (Ryder, Gild, Hohl et al., 2013).

To address the role of elevated TSH and the resultant TSHR/GNAS/cAMP signaling in this model, the TSH receptor was knocked out in the mice, resulting in significant attenuation of the phenotype. Similar findings were made when the Gsα subunit was knocked out the thyroid, suggesting that although BRAF signaling results in cellular hyperproliferation, TSH/GNAS/cAMP/PKA signaling is necessary for oncogenic transformation (Franco et al., 2011).

3.3.2 RTK FUSION PROTEIN MODELS

3.3.2.1 RET-PTC1 tumor models: The first model of the RET-PTC1 oncogene was published in 1996, in which the RET-PTC1 transgene was driven by the bovine Thyroglobulin (Tg) promoter. These mice developed thyroid overgrowth with malignant features in 100% of animals, with abnormalities detected as early as embryonic day 18 (Cho, Sagartz, Capen et al., 1999, Jhiang, Cho, Furminger et al., 1998, Jhiang, Sagartz, Tong et al., 1996). A similar model using the rat Tg promoter to drive transgene expression produced tumors with much longer latency, with about 30% of mice developing PTC by 16 months (Santoro, Chiappetta, Cerrato et al., 1996). Aside from the use of the Tg promoter from different animals, there were also strain differences between the mouse models which may account for the variations in phenotype. Efforts have also been made to develop a doxycycline-inducible allele of thyroid-specific RET-PTC1 expression, although the expression levels of the transgene were low and cancers did not develop (Knostman, Venkateswaran, Zimmerman et al., 2007).

To investigate the tyrosine sites in RET-PTC1 deemed important for transformation, transgenic mice were developed where key tyrosine sites were mutated (Buckwalter, Venkateswaran, Lavender et al., 2002). The mutated sites were pY294, pY404 and pY451 in RET-PTC1, corresponding to pY905, pY1015 and pY1062 in RET. In comparison to wild-type RET-PTC1, the rate of tumor formation was significantly decreased, demonstrating that although these sites play a major role in transformation, they were not completely necessary for the tumor formation. The signaling pathways may be redundant or complementary, where combined signaling leads to maximum transforming activity. More interestingly,

there was no correlation between the transgene expression level (for RET-PTC1) and the histological grade of the tumors. The authors opined that high level of RET-PTC1 expression may be necessary for the earlier stages of transformation but its continued presence is not necessary for the tumor formation. This finding is akin to similar findings in RET-PTC3 which are discussed below. In this scenario, the tumor microenvironment and additional factors may play a major supporting role in tumor formation.

3.3.2.2 RET-PTC3 tumor models: Similar to the approach with RET-PTC1, a transgenic mouse model of RET-PTC3 was created in which expression of the fusion oncogene was targeted to the thyroid using the bovine Tg promoter, leading to tissue-specific expression (Powell, Russell, Nibu et al., 1998). Mice developed thyroid neoplasia, with 69% of animals demonstrating thyroid hypercellularity by 3 months of age. The primary tumors in these animals had a generally solid appearance, with large regions devoid of follicles or papillae, suggesting similarity to the solid type PTC as had been reported in humans. Interestingly, in a small cohort of animals studied at advanced age, 2/6 (33%) were observed to exhibit lymph node metastasis, which were identified as thyroid cells by staining for both the RET-PTC3 transgene as well as for thyroglobulin. Although metastasis was observed, these tumors failed to grow in SCID or normal mice following transplantation.

3.2.2.3 TRK-T1 tumor models: To understand the role of theTRK1-T1 fusion protein in PTC development, constitutive thyroid-specific expression of this product was achieved using the bovine Tg promoter linked to the fusion cDNA (Russell, Powell, Cunnane et al., 2000). 100% of mice aged 7 months or above exhibited thyroid abnormalities such as hyperplasia, micro-papillary structures and further characteristics of differentiated carcinomas. Although 78% of mice developed solid tumors, they did not exhibit the nuclear abnormalities characteristic of PTC. Further, unlike the human PTC cases, where around 30% of the cases are accompanied by inflammation, these transgenic mice and PTCs were devoid of them. It was proposed that the T-cell tolerance against NTRK1 antigens due to the constitutive expression at early neonatal stage is responsible for this loss of immune response to the tumor. In this model, it was further observed that ablation of the p27 tumor suppressor gene increased PTC prevalence and decreased latency, suggesting that this tumor suppressor is an essential regulator of the PTC phenotype, at least due to TRK-T1 fusions (Fedele, Palmieri, Chiappetta et al., 2009).

3.3.3 Models of Ras activation—Because RAS mutations have been observed in about 10% of PTC, a number of labs have produced mice expressing activated isoforms Ras in the thyroid gland, either via transgenesis (Rochefort, Caillou, Michiels et al., 1996, Santelli, de Franciscis, Portella et al., 1993, Vitagliano, Portella, Troncone et al., 2006)or via targeted/cre-activated knock-in (Charles et al., 2011, Chen, Mitsutake, LaPerle et al., 2009, Miller, Yeager, Baker et al., 2009). Surprisingly, although these alleles may induce mild thyroid proliferation, PTC is not observed, at least with K-RAS and H-RAS. In mice with thyroid-specific expression of activated K-RAS^{G12D}, only rare follicular hyperplasia was seen (Charles et al., 2011, Miller et al., 2009, Santelli, de Franciscis, Chiappetta et al., 1993). In one study (Santelli et al., 1993), treatment of the mice with a goitrogen led 1/23 to develop “FTC’ based on the presence of abnormal appearing cells, although no local or distant

invasion was seen. H-RAS^{G12V}, which is thought to have lower signaling intensity than activated K-RAS alleles, has also been used to produce thyroid-specific expression of activated H-Ras (Chen et al., 2009, Rochefort et al., 1996). No thyroid tumors were observed, although the mice did appear to be more prone to mutations in thyroid cells carrying the activated RAS allele (Chen et al., 2009). In contrast to the observations with H-RAS or K-RAS, use of the bTG promoter to drive N-Ras^{Q61K} led to the production of invasive thyroid cancer in 40% of the mice (Vitagliano et al., 2006). Interestingly, tumors exhibited a mixed papillary/follicular morphology (see below), and about 25% of the tumors exhibited areas of de-differentiation. Although secondary mutations were not identified in the tumor samples, expression of the same oncogene into the PCCl3 rat thyroid cell line induced genomic instability, suggesting that secondary mutations may account for these observations. It is worth noting that this tumorigenic N-Ras line was generated as a transgenic, such that it may be less “physiological” than targeted knockins. For example, transgenics may incorporate multiple copies of the transgene, leading to abnormally high levels of transcript; alternately, the site of transgene integration may affect phenotype expression. Neither of these aspects has been fully explored in this model.

4. FOLLICULAR THYROID CANCER

4.1 Phenotype of human Follicular Thyroid Cancer

Follicular thyroid cancers (FTCs) are characterized by the retention of follicular or micro-follicular tumor architecture, and are often difficult to distinguish from follicular thyroid adenomas on fine needle aspiration biopsy, as they lack characteristic nuclear features as observed in PTC. FTC is more common in areas of dietary iodine deficiency and this pathology generally has a poorer prognosis than PTC due to reduced radioactive iodine effectiveness (Jonklaas, Sarlis, Litofsky et al., 2006, Schlumberger, 1998). FTC exhibits a pattern of hematogenous spread, where metastasis to the lung and other tissues is observed without intervening lymph node involvement; once disseminated, FTC rapidly becomes non-responsive to standard therapies (Ruegemer, Hay, Bergstralh et al., 1988, Zhang et al., 2014).

4.2 Molecular pathways and the Genetics of human FTC

Because FTC is much less common, there has been less work on the genetics of these tumors, including exclusion to date of this tumor subtype from the TCGA dataset. Single point mutations in a RAS gene family are observed in about 45% of FTC, most commonly N-Ras activating mutations at codon 61 (Nikiforov, 2004, Vasko, Ferrand, Di Cristofaro et al., 2003). The other commonly observed mutation in FTC is a gene fusion between the *PAX8* and PPARG genes, resulting in the so-called *PAX8-PPARG* fusion protein (PPFP) (DeLelllis, Lloyd, Heitz et al., 2004, Kroll, 2004, Lacroix, Lazar, Michiels et al., 2005). This mutation is observed in approximately 35% of FTC (Nikiforova and Nikiforov, 2009). Other mutations that are observed infrequently in FTC are amplification or mutation of *PIK3CA* encoding the catalytic subunit of the PI3-dependent protein kinase and mutation in *PTEN*, the dual purpose phosphatase which converts PIP3 to PIP2 (Nikiforova and Nikiforov, 2009). Mutations in the latter are observed in ~5% of sporadic FTC (Nagy, Ganapathi, Comeras et al., 2011).

Focus on FTC has also come from two inherited syndromes which include FTC as part of their tumor spectrum. Cowden syndrome (CS) is caused by heterozygous mutations in *PTEN*, and carries a standardized incidence ratio of 72 for thyroid cancer, including enhanced risk for both FTC and PTC; (Ngeow, Mester, Rybicki et al., 2011) however, because the population risk for FTC is much lower, the relative risk for FTC for these patients is significantly higher. Carney Complex (CNC) is another autosomal dominant tumor syndrome which includes an enhanced susceptibility to FTC. CNC is caused by mutations in *PRKAR1A*, encoding the type 1A regulatory subunit of PKA, leading to enhanced activation of PKA (Bertherat, Horvath, Groussin et al., 2009, Kirschner, Carney, Pack et al., 2000). These patients have about a 25% incidence of thyroid nodules, and about 10 % of these will undergo malignant degeneration, making the incidence of thyroid cancer about 2.5% overall in these patients. Analysis of spontaneous thyroid tumor shows that loss of PRKAR1A can occur in tumors, either through genetic or non-genetic means (Sandrini, Matyakhina, Sarlis et al., 2002).

4.3 MOUSE MODELS FOR FTC

4.3.1 SINGLE GENE MUTATION MODELS

4.3.1.1 Ras and Rap1 models: As described above, introduction of activated Ras alleles into the thyroid gland often fails to recapitulate thyroid carcinogenesis. These studies suggested that additional events are required for initiating FTC, as discussed below. The small G protein Rap1b has also been proposed to be important in thyroid tumorigenesis. To study this effect in mice, a transgenic mouse was developed which expressed a constitutively active $Rap1b^{G12V}$ isoform. (Ribeiro-Neto, Leon, Urbani-Brocard et al., 2004) Like the activated Ras mutants, these mice had a minimal phenotype. However, when $Rap1b^{G12V}$ mice were treated with a goitrogenic protocol (methimazole and perchlorate) for 1 year to invoke substantial TSH-TSHR signaling, they developed locally invasive FTC.

4.3.1.2 PPFP models: A transgenic mouse was generated which expressed the PAX8-PPARG fusion cDNA expressing the PPFP fusion gene in the thyroid. Surprisingly, these mice had a minimal phenotype at one year, with only mild thyroid hyperplasia observed in a subset of mice (Diallo-Krou, Yu, Colby et al., 2009). Use of the strong CAG promoter to drive PPFP resulted in significant thyroid hyperplasia by 6 months, although frank cancers were not seen. These authors argue that these high levels of transcript are more similar to what is observed in human thyroid tumors (Dobson, Diallo-Krou, Grachtchouk et al., 2011)

4.3.1.3 Pten KO models: Because of its connection to Cowden syndrome, it was expected that mice with KO of *Pten* would exhibit thyroid carcinogenesis. Mice that have complete KO of *Pten* exhibit embryonic lethality; however, heterozygotes are viable and fertile. Multiple groups generated such mice, and the thyroid phenotype was highly variable, ranging from thyroid cancer in over 50% of mice by 1 year to minimal-no thyroid disease (Di Cristofano, De Acetis, Koff et al., 2001, Podsypanina, Ellenson, Nemes et al., 1999, Stambolic, Tsao, Macpherson et al., 2000, Suzuki, de la Pompa, Stambolic et al., 1998).

As mouse phenotypes can be accented by tissue-specific KO compared to the heterozygote state, the effect of complete KO of *Pten* in the thyroid has also been described. The initial

description of the *Pten* thyroid specific KO suggested that these animals developed only mild follicular hyperplasia (Yeager, Klein-Szanto, Kimura et al., 2007), an observation which has also been made in our lab (Pringle, Vasko, Yu et al., 2014). However, subsequent studies from the di Cristofano labs have suggested that *Pten* loss is sufficient to drive metastatic FTC (Antico-Arciuch, Dima, Liao et al., 2010). Strain specific effects may account for these effects (Tiozzo, Danopoulos, Lavarreda-Pearce et al., 2012), although the explanation is not yet clear. Intriguingly, there was a gender bias where 52% of the $Pten^{-/-}$ female mice and only 12% of the males developed follicular adenomas by one year of age (Antico-Arciuch et al., 2010).

4.3.1.4 Prkar1a KO models: In the initial characterization study of $Prkar1a^{+/-}$ mice, thyroid tumors were observed in about 10% of mice aged up to 2 years, and the majority of such tumors were identified as solid-pattern thyroid carcinomas (Kirschner, Kusewitt, Matyakhina et al., 2005). Subsequent to that study, mice were generated with a tissue-specific KO of *Prkar1a* in the thyroid. Mice in that cohort developed locally invasive follicular thyroid cancer in about 45% of animals by 1 year of age (Pringle, Yin, Lee et al., 2012). Interestingly, the mice were also hyperthyroid due to TSH-independent activation of PKA signaling, although the role of excess thyroid hormone signaling in the cancer phenotype is currently unknown.

4.3.1.5 TRβ-PV mice: Another interesting mouse model of follicular thyroid carcinoma was developed by introduction of a dominant-negative mutation into the thyroid hormone receptor β (*THRB*) in a 129/Sv X C57BL/6J background strain. This mutation was called PV after the patient in whom it was first identified. This mutation caused loss of TRβ binding to active T3 hormone and hence no transcriptional activation. As there was no negative feedback by T3 on the hypothalamus and pituitary gland, TSH was upregulated and follicular cell hyperplasia was seen in these mice (Suzuki, Willingham and Cheng, 2002). At 4–5 months of age, invasive follicular thyroid carcinoma was seen in $THRB^{PV/PV}$ homozygotes (known as TRβ-PV mice). The majority of the mice had distant lung and heart metastases over the age of 5 months. These metastases have foci consisting of spindle-shaped cells. Even though this mouse model recapitulated human thyroid cancer, they exhibit elevated circulating T3 and T4 levels unlike human FTC (Kaneshige, Kaneshige, Zhu et al., 2000, Kato, Ying, Willingham et al., 2004). TRβ-PV mice were crossbred with $THRB^{-/-}$ mice, and $THRB^{PV/-}$ mice developed FTC spontaneously with lung metastasis (Kato et al., 2004).

It is well established that there is activation of AKT in thyroid tumors in humans (Ringel et al 2001). It has also been shown that PI3K/AKT pathway is activated in thyroids as well as metastatic lesions of TRβ-PV mice. This pathway has been shown to contribute to thyroid carcinogenesis and is a potential therapeutic target for FTC. When TRβ-PV mice were treated with LY294002, inhibitor of PI3K, there was a decrease in tumor growth, proliferation and increased survival (Furuya, Lu, Willingham et al., 2007). This was the first in vivo study for preclinical testing of PI3K inhibitor for FTC. Recently, TRβ-PV mice were bred with $Akt1^{-/-}$ mice (Saji, Narahara, McCarty et al., 2011). There was delayed thyroid cancer development and reduced tumor invasiveness in KO mice. At 12–15 months of age,

WT mice developed lung metastasis compared to KO mice. It was also shown that thyroid tumor development and progression was dependent on Akt1 in this mouse model system.

4.3.1.6 PIK3CA activation models: Phospho-inositide-3-kinase signaling is activated by many membrane receptors, and is normally suppressed by Pten. Although multiple isoforms exist, the major isoform is PIK3CA, which acts by through a signaling cascade that leads to Akt activation, causing increased proliferation and survival. Mutations or amplification of PIK3CA have been described in FTC, so efforts have been made to study the role of this kinase in mouse thyroid cancer. Using a tamoxifen-inducible system to express the constitutively active $PIK3CA^{H1047R}$ in the thyroid caused only a minimal phenotype, even after 1 year of oncogene expression, suggesting that activation of this enzyme by itself was insufficient to cause FTC (Charles, Silva, Iezza et al., 2014).

4.3.2 DUAL-HIT FTC MODELS—The genetic landscape of cancer is complicated, and many of the single gene models for FTC did not produce a cancer phenotype, with the exception of transgenic *N-Ras,* or knockouts of *Prkar1a and* Pten, as described above. However, in an effort to generate more pronounced cancer phenotypes, two-hit models for FTC have been generated, and these have generally been much more carcinogenic than the single-hit approaches.

Specifically, introduction of *Pten* KO into the PPFP model led to the presence of FTC with an aggressive phenotype within 5 months (Dobson et al., 2011). At the age of analysis, over 50% of a small cohort of animals (7/12) exhibited lung metastasis in a fashion similar to that observed in human cancers. Intriguingly, treatment of these mice with the PPARG agonist pioglitazone induced a marked anti-tumor effect where cells instead exhibited signs of adipocyte differentiation. To model cross-talk between the Ras/MAPK and PI3K pathways, a K-Ras mutant allele was conditionally expressed in thyroids of Pten knock out mice. Simultaneous activation of both pathways led to invasive and metastatic thyroid carcinoma (Miller et al., 2009). Similarly, creation of mice carrying double KO for *Prkar1a* and *Pten* exhibited 100% penetrance of FTC by 2 months of age, and about 30% of the mice developed frank lung metastasis (Pringle et al., 2014). Intriguingly, each of these models was associated with TSH-independent hyperthyroidism, an observation which has not yet been fully explored in these models.

5. MOUSE MODELS FOR ANAPLASTIC THYROID CANCER

5.1 Phenotype of ATC

Poorly differentiated and anaplastic thyroid carcinomas are relatively uncommon. These types of cancer are responsible for a few percent of all thyroid cancers in the United States, but unfortunately they have one of the worse prognoses of any solid malignancy (Santarpia, El-Naggar, Cote et al., 2008). The average age of patients diagnosed with ATC is 65 years or older with median survival less than 6 months. ATC is a very aggressive tumor than can exhibit significant local invasion (including the trachea and esophagus) as well as metastatic spread. Fortunately, it accounts for very small percentage of thyroid cancer (Nagaiah, Hossain, Mooney et al., 2011). ATC may arise spontaneously or progress from well differentiated thyroid carcinomas (DTC). The evidence for ATC originating from DTC (by

progressive malignant degeneration of a pre-existing differentiated PTC) includes coexistence of ATC and PDTC within the same tumor specimens (Nucera et al., 2009, Santarpia et al., 2008). Another theory suggests that undifferentiated thyroid carcinoma arises from the transformation thyroid stem cells (Todaro, Iovino, Eterno et al., 2010). Cancer stem cell markers CD133 and ALDH have been used to isolate thyroid cancer stem cells by flow cytometry, and those cells recapitulate the behavior of the parental tumor *in vivo* (Todaro et al., 2010). ATC is typically accompanied by cell proliferation and chromosomal instability, and morphological features including the gradual loss of papillary and follicular growth patterns, biphasic spindle, solid growth squamoid pattern, areas of necrosis and hemorrhage, and nuclear pleomorphism (Parenti, Salvatorelli and Magro, 2014, Salvatore, Nappi, Salerno et al., 2007). The two common signaling pathways have been implicated in ATC are PI3K and MAPK (Champa and Di Cristofano, 2015). Differentiated thyroid cancers often have mutations in these pathways and undifferentiated thyroid cancer has additional mutations such as p53. Likewise, anaplastic thyroid carcinoma (ATC) mouse models are relatively recent, and most of the significant advancements have been based on differentiated thyroid carcinomas mouse models (Parenti et al., 2014).

5.2 Single gene models for PDTC/ATC

Ledent and others developed an ATC mouse model by expressing SV 40 T-antigen under thyroglobulin promoter. Moderately to poorly differentiated thyroid adenocarcinomas were developed in older animals that progressed from hyperplasia at birth (Ledent, Dumont, Vassart et al., 1991). However, severe hypothyroidism was observed in this model that is unlike human thyroid carcinomas (Zhu and Cheng, 2012).

5.3 Double hit models

Another rationale for thinking that ATC arises out of PTC or FTC is the fact that most mouse models that develop ATC require two distinct genetic hits.

5.3.1 Pten + P53—Antico Arciuch et al. (2011) developed a mouse model combining two hallmarks of human ATC, Pten deletion and P53 inactivation. In 75% of the mice, pre-existing follicular hyperplasia and carcinoma developed aggressive and undifferentiated thyroid tumors by 9 months of age (Antico Arciuch, Russo, Dima et al., 2011). These tumors recapitulated features to human ATC including pleomorphism, epithelial-mesenchymal transition, aneuploidy, local invasion, and metastases (Antico Arciuch et al., 2011). Analyses of the *Pten/p53* model, as well as the *Pten/K-RAS* mutant described above, demonstrated upregulation of the mitotic kinase PLK1, suggesting common downstream pathways; Treatment with a PLK1 inhibitor produced some therapeutic benefit in both models (Russo, Kang and Di Cristofano, 2013)

5.3.2 RET-PTC1 + P53—ATC also harbors the highest frequency of *TP53* mutations(McFadden, Vernon, Santiago et al., 2014). It is well known that losing p53 protein alone is not sufficient to induce malignancy in the thyroid (Russo, Antico Arciuch and Di Cristofano, 2011). Earlier models of ATC were generated using transgenic mice expressing somatic rearrangements of the *RET* proto-oncogene (*RET-PTC1)* (Champa and Di Cristofano, 2015, La Perle, Jhiang and Capen, 2000). The *RET* proto-oncogene encodes a

transmembrane glycoprotein belonging to the receptor tyrosine kinase family. The fusion of the 3′-terminal kinase-encoding domain of RET to the 5′-terminal regions of genes forms a chimeric oncogene. *RET-PTC1* under thyroglobulin promoter in a $p53^{-/-}$ background developed anaplastic-like tumors in a model by La Perle et al. (La Perle et al., 2000). Although this model was useful, the mice developed extra-thyroidal tumors because of systemic deletion of p53 (Champa and Di Cristofano, 2015). Similar findings were made when RET-PTC3 transgenic mice were crossed into a *p53* knockout background; interestingly, it was shown that aggressive tumors in this model eventually lost expression of the RET-PTC3 fusion protein, indicating that late stage progression did not require continued expression of the oncogene (Powell, Russell, Li et al., 2001).

5.3.3 BRAF + P53—McFadden et al. showed that $BRAF^{V600E}$ Initiates PTC in the adult murine thyroid and p53 loss enables the progression to ATC (McFadden et al., 2014). Homozygous deletion of p53 further accelerated disease progression to PDTC and to ATC with a median survival of 6 months following tumor induction. 19% of mice developed microscopic metastases, which mimicked the invasive nature of human undifferentiated thyroid cancer. It was also shown that combination treatment with MEK and BRAF inhibitors results in enhanced antitumor activity as compared to treatment with a BRAF inhibitor alone (McFadden et al., 2014).

5.3.4 THRB + K-RAS—Adding $K\text{-}Ras^{G12D}$ mutation to TRβ PV, mice exhibited frequent anaplastic foci with complete loss of normal thyroid follicular morphology(Zhu, Zhao, Park et al., 2014). These mice had even poorer survival due to more aggressive thyroid tumors with capsular invasion, vascular invasion, and distant metastases.

5.3.5 B-RAF + PIK3CA—Although induction of constitutively active PIK3CA was unable to cause significant thyroid neoplasia, combining this mutation with B-RAF activation led to the production of ATC. However, the time course of tumorigenesis was relatively modest, with mice succumbing to their tumors between 6 and 12 month after induction of the oncogenes (Charles et al., 2014).

6. MEDULLARY THYROID CANCER

6.1 Phenotype of MTC

Medullary thyroid cancer most often presents as a palpable neck mass located within the thyroid on examination. It is one of the defining tumors of multiple endocrine neoplasia type 2 (MEN2), and individuals affected with the condition have essentially 100% penetrance of this cancer. Approximately 20% of cases of MTC are found as an inherited form of the disease, in which there is a germline activating mutation of the *RET* proto-oncogene. Of sporadic cases of MTC, between 40 and 50% of sporadic MTC cases are found to carry RET *mutations (*Elisei, Cosci, Romei et al., 2008). Normal C-cells secrete calcitonin, and blood levels of this hormone are a good marker for MTC. Because this is a neuroendocrine-derived tissue, patients can develop significant diarrhea, either from calcitonin itself or from other gut peptides secreted by the tumors. Tumors can also secrete other neuropeptides, including ACTH, leading to ectopic Cushing syndrome. MTC also is a metastatically aggressive

tumor, with frequent bulky liver involvement, as well as involvement of local lymph nodes and other distant organs.

6.2 MOUSE MODELS FOR MTC

6.2.1 RET transgenic models for MTC—Since the discovery of mutations in the *RET* gene causing multiple endocrine neoplasia type 2 (MEN2) and Familial MTC (FMTC), the creating of a transgenic mouse expressing mutated Ret in the thyroid was an obvious target. Mice carrying RET^{C634R} under the control of the rat calcitonin/calcitonin gene related peptide (Ct/CGRP) promoter were described in 1997 (Michiels, Chappuis, Caillou et al., 1997). Mice from three independent lines were studied, and they expressed the transgene primarily in the thyroid and the adrenal gland, although low-level expression was also observed in the brain and kidney. Mice developed C-cell hyperplasia as early as 3 weeks of age in the line with highest expression of the transgene, and MTC was observed by 8 months. Lines with fewer copies/lower expression of the transgene had a much longer latency for the development of tumors.

In another effort, the same C634R RET allele was driven the Moloney murine leukemia virus (MoMLV) LTR. Expression of this transgene was less restricted, but expression in C-cells was sufficient to drive C-cell hyperplasia as well as tumorigenesis in the mammary and parotid glands, although other expressing tissues did not develop tumors. To date, two mice have been made using the RETM918T MEN2B mutation, although neither was targeted specifically to the thyroid (de Graaff, Srinivas, Kilkenny et al., 2001, Sweetser, Froelick, Matsumoto et al., 1999). A constitutive knock-in of RETM918T (M919T in mice) was made, demonstrating many of the features of MEN2, including C-cell hyperplasia, pheochromocytomas, and sympathetic ganglioneuroma-like tumors (Smith-Hicks, Sizer, Powers et al., 2000). MTCs were not observed in this model. In a transgenic model which used the human calcitonin gene promoter to express human RET 918T, MTC were observed, but only with an incidence of 13% in mice older than 11 months. Use of a similar construct to drive expression of WT human RET did not result in any tumorigenesis (Acton, Velthuyzen, Lips et al., 2000).

6.2.2. Mutations in cell-cycle control genes—The *Rb1* tumor suppressor was one of the first mammalian genes knocked out of the mouse as a model for the well-established role of Rb1 as causing Retinoblastoma in humans. This condition, which is inherited as an autosomal dominant trait, is due to somatic loss of the *RB1* gene in retinoblastomas, and was one of the paradigms of the inheritance of tumor suppressor genes as causing inherited human cancers. In mice, complete KO of *Rb1* caused embryonic lethality. However, heterozygous mice are born and are tumor prone, although they do not develop retinoblastomas.

Interestingly, mice heterozygous for *Rb1* mutations develop intermediate pituitary lobe tumors with high frequency. Subsequently, it was observed that these mice also develop MTC with high frequency. In the Jacks' labs, MTC was observed in up to 75% of animals (Williams, Remington, Albert et al., 1994), and similar findings were made in Wen-Hwa Lee's lab, where tumors were observed in 68% of morbid mice. However, in a third

independent lab, MTCs' were not observed at all in $Rb^{+/-}$ animals (Harvey, Vogel, Lee et al., 1995). Studies of genetic interaction indicated that introduction of a null allele for *Tp53* into the $Rb^{+/-}$ line (i.e. $Rb^{+/-}$;$Tp53^{+/-}$mice) enhanced the generation of MTC, typically associated with loss of the WT *Rb1* and *Tp53* alleles (Harvey et al., 1995, Nikitin, Liu, Flesken-Nikitin et al., 2002, Williams et al., 1994). However, $Rb^{+/-}$; $Tp53^{-/-}$ mice actually exhibited reduced MTC, often because they were dying from other tumors before the onset of this phenotype. In one study analyzing the mechanism of this observation, it was observed that MTCs arising in this model frequently exhibited *Ret* mutations, suggesting that enhanced somatic mutation rate may play a role in the process (Coxon, Ward, Geradts et al., 1998).

Phospho-Rb is known to exert its tumor suppressor effects through interactions with the Cyclin dependent kinases (CDKs), which are also inhibited by a 7-gene family known as the CDK inhibitors (CDKNs). Analysis of KO mice for CDKN2C (also known as $p18^{INK4C}$) demonstrated the spontaneous development of C-cell hyperplasia in 10–14% of mice by one year of age (Franklin, Godfrey, O'Brien et al., 2000, van Veelen, van Gasteren, Acton et al., 2008). Mice lacking CDKN1B ($p27^{KIP1}$, which is also associated with multiple endocrine neoplasia syndrome in humans and in a spontaneous rat model)(Pellegata, Quintanilla-Martinez, Siggelkow et al., 2006) also develop low frequency (<5%) C-cell hyperplasia (Franklin et al., 2000). Interestingly mice lacking Cdkn2c who also have ablation of one or both *Cdkn1b* alleles develop C-cell lesions in over 50% and 85% of mice by 1 year of age, respectively. Cancers are uncommon, possibly because the mice develop aggressive pituitary tumors that do not enable long-term follow up of the thyroids. In contrast, ablation of Cdkn1a ($p21^{Cip1/Waf1}$) does not induce C-cell proliferation, either by itself or in combination with p18 KO (Franklin et al., 2000). Mutations in p18 have been found in a population of MTC in human patients with RET mutations (van Veelen, Klompmaker, Gloerich et al., 2009), and the ability of p18 mutations to facilitate RET-mediated MTC tumorigenesis has also been observed in a mouse model (van Veelen et al., 2008).

Lastly, in a more recent model, direct overexpression of the Cdk5 co-factor p25 (encoded by *CDK5R1)* in C-cells (under the control of the neuron-specific enolase promoter!) drove the development of MTC (Pozo, Castro-Rivera, Tan et al., 2013). In this inducible system, overexpression of p25 caused tumorigenesis by 16 weeks, and mice did not survive past 30 weeks. Analysis of the mechanism demonstrated that p25 overexpression led to enhanced Cdk5 kinase activity, leading to phosphorylation and inactivation of Rb.

7. CONCLUSIONS AND FUTURE DIRECTIONS

Although the prognosis for most patients with thyroid cancer remains good, there has been recent focus on patients with more aggressive forms of the disease, including those with metastatic papillary, follicular, and medullary thyroid cancers, as well as all patients with anaplastic disease. Genetic analysis and mouse modeling have both reached the point where the mutations that drive thyroid cancer have been generated and studied in the mouse. This confluence provides the opportunity for significant advancement in the development of therapeutic strategies. Although genetic models generally take longer to develop tumors and to analyze therapy effects, they have significant advantages over other techniques. First,

these studies are carried out in the intact animal without the need for immunosuppression as required for xenograft models. Second, drug effects can be studied in the intact animal, such that unexpected toxicity (or benefit) can be identified before moving to human studies. Finally, genetic mouse models can be used for analysis at any stage of progression, up to and including metastatic disease for some models. It is not an unreasonable expectation that current models will be subject to further refining, but that the major thrust for these studies over the next few years will be in the development of new therapies to treat patients with thyroid cancer.

Acknowledgments

We would like to acknowledge the collegiality and valuable discussions with the members of the OSU Thyroid Cancer Group, including Drs. M. Ringel, A. de la Chapelle, S. Jhiang, K. La Perle, and M. Saji. We would also like to thank Dr. D. Huk for her careful reading and thoughts on the manuscript. This work was supported in part by Grants CA170249 (to LSK) and CA124570 (Matthew Ringel, PI), and by grant CA016058 to the Ohio State University Comprehensive Cancer Center.

Abbreviations

PTC	Papillary thyroid cancer
MTC	Medullary thyroid cancer
FTC	Follicular thyroid cancer
RAI	radioactive iodine
TCGA	The Cancer Genome Atlas
Tg	Thyroglobulin
TSH	Thyroid stimulating hormone
MEN2	Multiple Endocrine Neoplasia, type 2

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Highlights

- Mutations which cause thyroid cancer in humans have been modeled in mice
- Multiple hits are required for metastatic follicular thyroid or anaplastic cancer
- Medullary thyroid cancer is modeled by mutations in Rb or cell cycle control genes
- Genetic mouse models are good pre-clinical tools for the development of new therapies

TABLE 1

Single gene models for non-medullary thyroid cancer in the mouse*

Gene	Mutation	Human phenotype	Mouse phenotype	Alleles**
BRAF	V600E	PTC (usually cPTC or fvPTC)	PTC	TX KI KI-I TX-I
RET-PTC1	Fusion gene	PTC	PTC (non-invasive)	TX
RET-PTC3	Fusion gene	PTC	PTC (non-invasive)	TX
TRK-T1	Fusion gene	PTC	PTC (non-invasive)	TX
H-RAS	G12D/G12V	PTC or FTC	hyperplasia	TX/KI
K-RAS	G12D	PTC or FTC	hyperplasia	TX/KI
N-RAS	Q61K	PTC or FTC	PTC/FTC	TX
Rap1b	G12V	ND	hyperplasia	KI-I
PAX8-PPARG	Fusion gene	FTC	hyperplasia	TX
PTEN	Knockout	FTC or PTC	FTC (likely strain dependent)	Het KO
Prkar1a	Knockout	FTC or PTC	FTC	Het KO
$THRB^{PV/PV}$	PV (fs443)	RTH (het)***	FTC	KI (homo)
PIK3CA	H1047R	FTC (?)	Minimal hyperplasia	KI-I
SV40 T-ag	Viral gene	--	ATC	TX

* References for each of the models can be found in the text

** TX: Transgenic, KI: Knock-in, KI-I: Inducible Knock-in, TX-I: Inducible transgenic, Het: Conventional heterozygote, KO: Knockout (tissue-specific)

*** RTH: Resistance to thyroid hormone.

Note that patients are heterozygous for the mutation. The phenotype is observed in homozygous mice

Table 2

Multi-hit models for NMTC in the mouse*

Gene 1	Gene 2	Phenotype
PPFP TX**	*Pten* KO	Metastatic FTC
K-Ras^{G12D} KI	*Pten* KO	Metastatic FTC
Prkar1a KO	*Pten* KO	Metastatic FTC
THRB$^{PV/PV}$	*K-Ras* KI	ATC
RET-PTC1 TX	*Tp53* KO	ATC
RET-PTC3 TX	*Tp53* KO	ATC
BRAF^{V600E} TX	*Tp53* KO	ATC
Pten KO	*Tp53* KO	ATC
BRAF^{V600E} TX	*PIK3CA*^{H1047R} *KI-I*	ATC

*References for each of the models can be found in the text

**Abbreviations as in Table 1

Table 3

Mouse Models for MTC*

Gene	Mutation	Alleles
RET (MEN2A)	C643R	TX**
RET (MEN2B)	M918T	TX KI
Rb	KO	Het
Cdkn2c (p18)	KO	Homo
Cdkn1b (p27)	KO	Homo
CDK5R1	p25-GFP fusion protein	TX-I

* References for each of the models can be found in the text

** Abbreviations as in Table 1