Thyroid-specific ablation of the Carney complex gene, *PRKAR1A*, results in hyperthyroidism and follicular thyroid cancer

Daphne R Pringle¹, Zhirong Yin¹,†, Audrey A Lee¹, Parmeet K Manchanda¹, Lianbo Yu², Alfred F Parlow³, David Jarjoura², Krista M D La Perle⁴ and Lawrence S Kirschner¹,5

¹Department of Molecular, Virology, Immunology, and Medical Genetics, The Ohio State University, 420 West 12th Avenue, Tzagournis Research Facility 544, Columbus, Ohio 43210, USA
²Center for Biostatistics, The Ohio State University, Columbus, Ohio 43210, USA
³National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, California 90509, USA
⁴Department of Veterinary Biosciences, The Ohio State University, Columbus, Ohio 43210, USA
⁵Division of Endocrinology, Diabetes and Metabolism, The Ohio State University, 420 West 12th Avenue, Tzagournis Research Facility 544, Columbus, Ohio 43210, USA

(Correspondence should be addressed to L S Kirschner at Department of Molecular, Virology, Immunology, and Medical Genetics, The Ohio State University; Email: lawrence.kirschner@osumc.edu)

†Z Yin is now at Department of Otolaryngology, Vanderbilt University, Nashville, Tennessee 37232, USA

Abstract

Thyroid cancer is the most common endocrine malignancy in the population, and the incidence of this cancer is increasing at a rapid rate. Although genetic analysis of papillary thyroid cancer (PTC) has identified mutations in a large percentage of patients, the genetic basis of follicular thyroid cancer (FTC) is less certain. Thyroid cancer, including both PTC and FTC, has been observed in patients with the inherited tumor predisposition Carney complex, caused by mutations in *PRKAR1A*. In order to investigate the role of loss of *PRKAR1A* in thyroid cancer, we generated a tissue-specific knockout of *Prkar1a* in the thyroid. We report that the resulting mice are hyperthyroid and developed follicular thyroid neoplasms by 1 year of age, including FTC in over 40% of animals. These thyroid tumors showed a signature of pathway activation different from that observed in other models of thyroid cancer. In vitro cultures of the tumor cells indicated that *Prkar1a*-null thyrocytes exhibited growth factor independence and suggested possible new therapeutic targets. Overall, this work represents the first report of a genetic mutation known to cause human FTC that exhibits a similar phenotype when modeled in the mouse. In addition to our knowledge of the mechanisms of human follicular thyroid tumorigenesis, this model is highly reproducible and may provide a viable mechanism for the further clinical development of therapies aimed at FTC.

*Endocrine-Related Cancer* (2012) 19 435–446

Introduction

Epithelial thyroid cancer (i.e. nonmedullary thyroid cancer) is the most common endocrine malignancy in the general population and rates of thyroid cancer continue to rise in the United States beyond what has been predicted from improved case ascertainment (Aschebrook-Kilfoy et al. 2010). Well-differentiated nonmedullary thyroid cancer is divided into papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) based on histological criteria. Although PTC is the more common subtype, patients with FTC have a poorer prognosis owing to the tendency of this tumor to behave more aggressively, including local and vascular invasion and distant metastases (Besic et al. 1999).
Although there is a wealth of information regarding the molecular basis of PTC (reviewed in Nikiforov (2011)), less is known about the genetics of FTC. Analysis of sporadic tumors has identified mutations in RAS in a small subset of tumors, whereas other investigators have demonstrated activating fusions between the PAX8 and PPARG transcription factors (Lacroix et al. 2005, Niepomnisczce et al. 2006). As PAX8 is required for thyroid development, it has been hypothesized that the PPARG–PAX8 fusion protein causes cancer by activating aberrant gene transcription.

Analysis of genetic syndromes that include FTC is another potential source for the identification of the molecular mechanisms contributing to carcinogenesis. FTC is associated with two tumor predisposition syndromes, Carney complex (CNC) and Cowden syndrome (CS). CNC (OMIM #160980) is characterized by spotty skin pigmentation, myxomas, endocrine tumors, and schwannomas (Stratakis et al. 2001). In a recent large series, benign thyroid adenomas were observed in 25% of cases, and thyroid cancer, either PTC or FTC or both, was found in 2.5% of patients, including being the cause of death in one patient (Bertherat et al. 2009). CNC is caused by inactivating mutations in PRKAR1A (Kirschner et al. 2000), which encodes the type 1a regulatory subunit of the cAMP-dependent protein kinase (protein kinase A, PKA), and mutations of this gene have been observed in sporadic cases of thyroid cancer (Sandrini et al. 2002). CS (OMIM #158350) is characterized as a multiple hamartoma syndrome and includes brain and breast cancer in addition to FTC. This syndrome is caused by inactivating mutations in PTEN, a dual-specificity phosphatase that negatively regulates the PI3 kinase/AKT pathway. Mutations in this gene have been detected in a variety of advanced sporadic cancers (reviewed in Hollander et al. (2011)) and in ~5% of FTCs (Nagy et al. 2011).

Our laboratory has been interested in studying the mechanism of tumorigenesis associated with mutations in PRKAR1A and its mouse homolog Prkar1a. We have previously reported that Prkar1a+/− mice are prone to tumors in cAMP-responsive tissues, including the thyroid (Kirschner et al. 2005). Tissue-specific deletion of Prkar1a in cAMP-responsive tissues such as GH-producing pituitary cells and Schwann cells can result in benign tumorigenesis associated with increased intracellular PKA activity (Jones et al. 2008, Yin et al. 2008b).

In this paper, we sought to extend these observations by generating a tissue-specific knockout (KO) of Prkar1a in the thyroid. We demonstrate that loss of Prkar1a in the thyroid gland leads to thyroid hyperfunction and the formation of FTC. This new model of FTC adds to our understanding of the molecular mechanisms involved in FTC development and may help to guide the development of new therapies.

**Materials and methods**

**Animal studies**

Mice were maintained in a sterile environment under 12 h light:12 h darkness cycles, and animal experiments were carried out in accordance with the highest standards of animal care under an IACUC-approved protocol. The generation of Prkar1a<sub>loxP/loxP</sub> and thyroid peroxidase-cre (Tpo-cre) animals has been described (Kusakabe et al. 2004, Kirschner et al. 2005). Prkar1a<sub>loxP/loxP</sub> and Tpo-cre mice were mated in order to generate Tpo-cre; Prkar1a<sub>loxP/loxP</sub> (R1a-TpoKO) mice.

**Histology**

Immunohistochemistry experiments were performed as described previously (Jones et al. 2008) with the following antibodies: Akt (9272), pAkt<sup>Ser473</sup> (3787), Erk1/2 (Mapk1, Mapk3) (EPHB2) (9102), pErk<sup>Thr202/Tyr204</sup> (9101), and cleaved caspase 3 (9661) (Cell Signaling Technology, Danvers, MA, USA); and Ki-67 (550609) (BD Biosciences, San Jose, CA, USA).

**Western blotting**

Proteins from mouse tissues were prepared and run on SDS–PAGE gels and transferred to nitrocellulose, blocked with 5% nonfat dry milk or BSA, and probed with the indicated antibodies. Antibodies used in this study were as follows: Akt (9272), pAkt<sup>Ser473</sup> (9271), Erk1/2 (Mapk1, Mapk3) (9102), pErk<sup>Thr202/Tyr204</sup> (9101), pStat3<sup>Tyr705</sup> (9145P), Stat3 (9132), pCreb<sup>Ser133</sup> (9198), and Creb (9197) (Cell Signaling Technology); Pcna (sc-56) and Spot14 (Thrsp) (sc-137178) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and Ki-67 (550609) (BD Biosciences, San Jose, CA, USA).

**Microarray**

RNA was isolated from the thyroids of 1-year-old wild-type (WT) and R1a-TpoKO mice using the Qiagen miRNeasy kit according to the manufacturer’s instructions (Qiagen). cDNA was made using the Bio-Rad iScript cDNA Synthesis Kit (Bio-Rad Laboratories). cDNA samples were hybridized to the Affymetrix Mouse Exon 1.0 ST Array Chip.
(Affymetrix, Santa Clara, CA, USA). Pathways were analyzed using Ingenuity Pathways Analysis Software (IPA; Ingenuity Systems, www.ingenuity.com).

Quantitative real-time PCR

RNA was isolated from mouse thyroids or cultured R1a-TpoKO cells and converted to cDNA with the Bio-Rad iScript cDNA Synthesis Kit (Bio-Rad Laboratories). cDNA was subject to qRT-PCR using the iQ SYBR Green Supermix Kit (Bio-Rad Laboratories) as per the manufacturer’s instructions. Reactions were each performed in triplicate. Primers were as follows: androgen receptor (Ar); 5'-GGACCATGTTTACCCATCG, 3'-TCGTTTCTCGTGGCACATA; uncoupling protein 1 (Ucp1); 5'-GGGCCCTTGAAACACAAAA, 3'-GTCGGTCTCTCTTTGTTGTA; C3; 5'-AAGCATCAACACACCCAACA, 3'-CTTGAGCTCCATTCGTGACA; Bcl3; 5'-TTACTCTACCCCCAGATGG, 3'-CCAAGCTTGAAAAGGCTGAG; Nfil3; 5'-CGGAAGTTGCATCTCAGTCA, 3'-CCAAGCTTGAAAAGGCTGAG; Icam1; 5'-AGGAAGTTGCATCTCAGTCA, 3'-CCAAGCTTGAAAAGGCTGAG.

Primary cell culture

Primary culture of thyroid cells was performed as described previously (Jeker et al. 1999). Briefly, cells were cultured on poly-d-lysine-coated plates in 5% fetal bovine serum in F12 media supplemented with l-glutamine, nonessential amino acids, sodium bicarbonate, and 6H hormone mix (TSH, insulin, hydrocortisone, glycy1-histidyl-L-lysine, transferrin, and somatostatin). All experiments were performed on cells through passage 20 or less. Inhibitors used were as follows: 10 μM LY294002 and 10 μM U0126 (Cell Signaling Technology); and 5 μM myristoylated PKI (Invitrogen), 10 μM HO-3867 (the generous gift of Dr P Kuppusamy, The Ohio State University). Cells were incubated in the presence of indicated inhibitors or media for 48 h (fresh inhibitors were added every 24 h), followed by MTT assays performed using the MTT Cell Proliferation Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions. All experiments were performed in triplicate.

Statistical analysis

All data, except microarray results, were analyzed via Student’s t-test using StatCrunch Software (www.statcrunch.com); P values <0.05 were considered significant.

For microarray results, signal intensities were quantified by Affymetrix software. Background correction and quantile normalization were performed to adjust technical bias, and gene expression levels were summarized using the RMA method (Irizarry et al. 2003). A filtering method based on percentage of arrays above noise cutoff was applied to filter low expression genes. A linear model was used to detect differentially expressed genes. In order to improve the estimates of variability and statistical tests for differential expression, a moderated t-statistic with variance smoothing was used for this study (Jeker et al. 2011). The significance level was adjusted by controlling the mean number of false positives (Gordon et al. 2007).

Results

Deletion of Prkar1a in the thyroid results in hyperthyroidism and follicular thyroid neoplasia

In order to generate a thyroid-specific deletion of Prkar1a, we crossed mice harboring the Tpo-cre transgene with Prkar1aloxP/loxP animals to obtain Tpo-cre, Prkar1aloxP/loxP animals, hereafter referred to as R1a-TpoKO. R1a-TpoKO mice and WT littermates were followed up to 1 year of age and then killed. Blood was collected for analysis of thyroid function, and the thyroids were harvested for gross and histological examination. Thyroid hormone (T4) levels in the R1a-TpoKO mice (n=12) were markedly elevated compared with the controls (n=12) (8.3 ± 4.4 μg/dl for KO vs 3.2 ± 1.1 μg/dl in WT, P=0.0009), indicating biochemical hyperthyroidism. TSH levels were also measured and were found to be lower in the R1a-TpoKO mice (157.25 ± 54.13 ng/ml in KO vs 353 ± 640 ng/ml in WT), although the differences were not statistically significant due to wide variations in the WT. While it is somewhat surprising that TSH levels were not significantly suppressed in these animals, it is a reasonable result given the relatively reduced sensitivity of the assay at the low end of the measurement range. To further show functional hyperthyroidism in these animals, body weights of WT and R1a-TpoKO animals at 1 year were compared. Although R1a-TpoKO females (n=5) were on average smaller than their WT counterparts (n=5) (28.75 ± 6.32 g for KO vs 37.91 ± 8.87 g for WT, P=0.0968), the difference did not reach statistical significance. However, these data along with the elevated T4 levels in these animals lead us to conclude that the R1a-TpoKO animals are functionally hyperthyroid.
Examination of the thyroids of these animals showed enlargement of the thyroids of 100% in R1a-TpoKO mice (Fig. 1A and B). At the histological level, the thyroids exhibited increased cellularity but retained a follicular pattern of growth (Fig. 1C and D). In order to determine whether the neoplastic thyroids contained foci of thyroid cancer, we studied them for the presence of cytological changes, as well as local invasiveness and distant metastasis. There were no morphologic or nuclear changes to suggest PTC in the specimens. However, we detected invasion through the thyroid capsule and in the surrounding tissues in 10/23 (43%) of the thyroids studied (Fig. 1F). While local invasion was detected in 43% of animals, none of the tumors examined showed widely invasive or angioinvasive behavior. Examination of lymph nodes in the neck, lungs, liver, and brain failed to detect the presence of distant metastases in any tissue studied. Based on the histological data, we conclude that R1a-TpoKO mice develop FTC at a rate of 43% by 1 year of age.

**R1a-TpoKO tumors exhibit increased proliferation independent of the AKT and ERK pathways**

We next attempted to elucidate signaling pathways that promote tumorigenesis in this model. Because RAS/RAF/ERK and PI3K/AKT signaling have been reported to be important tumorigenic pathways in FTC, we first attempted to determine whether these pathways were activated in the R1a-TpoKO tumors.
As shown in Fig. 2A, neither Akt nor Erk showed activation in these tumors, as judged by the absence of the phosphorylated (activated) isoforms of the proteins. The western blotting data were confirmed with immunohistochemical staining of tissue sections, which was also negative for the phosphorylated proteins (data not shown). In attempting to identify other pathways that may be associated with carcinogenesis, we identified activation of Stat3 as a consistent change found in the tumors (Fig. 2A).

We also examined the proliferative and apoptotic indices of these tumors using immunohistochemical staining. Ki-67 staining showed that R1a-TpoKO tumors had a much higher number of proliferating cells than WT thyroids (Fig. 2B and C, quantified in D). We also found that cleaved caspase 3 staining of these tumors showed no change in apoptosis rates compared with WT (Fig. 2E and F, quantified in G). These results indicate that the formation of these tumors is driven primarily by an increase in proliferation and not due to a failure of apoptosis.

**Microarray analysis identifies enhanced proliferation and altered differentiation pathways in R1a-TpoKO tumors**

In order to further elucidate the pathways involved in tumorigenesis in this model, we performed microarray analysis on thyroids from 1-year-old WT (n = 8) and R1a-TpoKO (n = 8) animals. The complete list of genes (Supplementary Table 1, see section on supplementary data given at the end of this article) was then analyzed using IPA Software, which identified transcriptional networks altered in the tumors. Table 1 lists the four networks showing the highest scores for alterations in the tumors, and the genes that comprise each network are included in Supplementary Table 2, see section on supplementary data given at the end of this article.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Score</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34</td>
<td>Cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>Cardiovascular system development and function, cellular development, cellular movement</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>Reproductive system development and function, cellular development, cellular growth and proliferation</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>Cardiovascular system development and function, cancer, developmental disorder</td>
</tr>
</tbody>
</table>

Additionally, pathways related to development were associated with all the four of these highly altered networks, suggesting that differentiation may also be altered in these tumors. These data are consistent with our previously published work, which suggests that PKA is involved in differentiation and development in a number of cAMP-responsive tissues (Nadella et al. 2008, Yin et al. 2008a, Jones et al. 2010). However, genes associated with thyrocyte terminal differentiation, including thyroid peroxidase, thyroglobulin, and the sodium iodide symporter, were expressed at normal or even higher levels than WT in the R1a-TpoKO tumors (Supplementary Table 4, see section on supplementary data given at the end of this article). These data highlight the fact that thyrocyte differentiation is inherently more complicated than the expression levels of these genes may indicate.

**Microarray analyses point to TRβ as a modulator of tumorigenesis**

In addition to the networks described earlier, the IPA analysis identified a signaling network that involved the thyroid hormone receptor β (TRβ, encoded by Thrb) (Fig. 3). Although TRβ mRNA expression was not altered in the microarray study, several genes known to be targets of TRβ signaling were identified, including mRNAs encoding the Ar, Spot14 (Thrsp), and Ucp1. In order to validate this result, we performed quantitative real-time PCR and western blot analyses on these targets (Fig. 4). qRT-PCR confirmed that both Ar and Ucp1 were downregulated in the R1a-TpoKO thyroids compared with WT (Fig. 4A and B). Western blot analysis of Spot14 indicated a reduction and/or loss of protein expression in the R1a-TpoKO thyroids (Fig. 4C).
Primary cells cultured from R1a-TpoKO thyroids exhibit growth factor independence but require STAT3 signaling

In order to provide an in vitro model system for the study of signaling mechanisms, we established primary cultures of cells derived from R1a-TpoKO thyroid tumors. More than 90% of cultured cells expressed thyroglobulin at a level similar to FRTL5 cells through passage 15 (Supplementary Figure 1, see section on supplementary data given at the end of this article), confirming their ability to proliferate and retain thyroid differentiation in vitro. Because WT mouse thyroid cells do not proliferate in vitro, we used the well-established FRTL5 rat thyroid cell line as a control for these studies (Ambesi-Impiombato et al. 1980).

Primary thyroid cultures typically require the inclusion of TSH and insulin in the medium as growth factors to enable cell proliferation. As shown in Fig. 5A, removal of TSH, insulin, or both from the medium results in significant reduction of proliferation in FRTL5 cells when compared with cells grown in complete (6H) medium. In contrast, R1a-TpoKO cells retained their capacity to proliferate independent of the presence of these growth factors (Fig. 5B). Expression of Tshr on both primary tumors and tumor cells grown in culture was validated (Supplementary Figure 1C) in order to confirm that this TSH-independent growth was not a consequence of loss of the receptor.

Next, we sought to assess the effects of pharmacological inhibitors on cell proliferation. Based on the
data shown earlier, we hypothesized that inhibition of the AKT or MEK/ERK pathways would not lead to growth inhibition, while inhibition of PKA or STAT3 would slow cell growth. Surprisingly, LY294002, an inhibitor of AKT, significantly reduced cell proliferation compared with vehicle (Fig. 5D). Conversely, myristoylated PKI, a cell permeable specific inhibitor of PKA, failed to reduce cell growth (Fig. 5D). A novel STAT3 inhibitor, HO-3867 (Selvendiran et al. 2010) was able to significantly reduce cell proliferation at the highest dose. In order to compare our primary tumor cells with normal thyroid cells, we also examined the growth of FRTL5 cells in the presence of these inhibitors (Fig. 5C). All these inhibitors were able to inhibit growth of FRTL5 cells at the highest concentrations. Western blot analysis of pAkt, pErk, and pCreb were performed to validate the inhibition of their intended targets (Supplementary Figure 2, see section on supplementary data given at the end of this article).

In order to confirm that inhibition of cell growth by HO-3867 was due to Stat3-mediated effects, we first confirmed that treatment with HO-3867 reduces phosphorylation of Stat3 as shown in Fig. 6A.

---

**Figure 4** qRT-PCR and western blot analyses confirm possible involvement of TRβ signaling. qRT-PCR experiments of Ar (A) and Ucp1 (B) in the R1a-TpoKO (KO) thyroids compared with wild-type (WT) thyroids (n=3). (C) Western blot analysis of Spot14 (Thrsp) in WT and R1a-TpoKO thyroids.

**Figure 5** Cells derived from R1a-TpoKO thyroids show differing responses from normal thyroid cells in the absence of growth factors and presence of inhibitors. (A) MTT assay of FRTL5 cells, treated with indicated media, were grown in complete medium (6H) or in medium lacking insulin (5H-INS), TSH (5H-TSH), or both (4H). (B) R1a-TpoKO cells, treated as in A. (C) FRTL5 cells treated with the indicated inhibitors at the following concentrations: 10 μM LY294002, 10 μM U0126, 5 μM PKI, and 1, 3, and 10 μM HO-3867 as indicated. (D) R1a-TpoKO cells, treated as in C. In A and B, *P<0.05 compared with cells grown in 6H medium; in C and D, *P<0.05 compared with vehicle treatment.
Additionally, we performed quantitative real-time PCR analysis of known Stat3 target genes (Dauer et al. 2005). Figure 6B shows that of the four Stat3 target genes (C3, Bcl3, Nfil3, and Icam1), three were significantly downregulated after 48 h of treatment with HO-3867 compared with cells treated with vehicle. Together, these data confirm the inhibition of Stat3’s transcriptional activity by HO-3867.

**Figure 6** Treatment of R1α-TpoKO cells with a Stat3 inhibitor reduces expression of Stat3 target genes. (A) Protein lysates from FRTL5 and R1α-TpoKO cells treated with the indicated inhibitors for 48 h were probed with antibodies against pStat3 and Stat3 as indicated. (B) qRT-PCR experiments of C3, Bcl3, Nfil3, and Icam1 in R1α-TpoKO cells treated with HO-3867 compared with vehicle treatment. *P<0.0001 compared with vehicle treatment.

Additionally, we performed quantitative real-time PCR analysis of known Stat3 target genes (Dauer et al. 2005). Figure 6B shows that of the four Stat3 target genes tested (C3, Bcl3, Nfil3, and Icam1), three were significantly downregulated after 48 h of treatment with HO-3867 compared with cells treated with vehicle. Together, these data confirm the inhibition of Stat3’s transcriptional activity by HO-3867.

**Discussion**

As rates of thyroid cancer continue to rise, it remains relevant to identify new murine models that can lead to insights into the mechanisms of thyroid cancer formation as well as serve as preclinical drug testing models. In this study, we observed that thyroid-specific KO of Prkar1a leads to hyperthyroidism and thyroid cancer. R1α-TpoKO mice develop large tumors with 43% being classified as FTC (total n = 23). Notably, no distant metastases were seen in any of our animals, suggesting that another genetic mutation may be necessary to elicit metastases in the context of Prkar1a mutation in the thyroid.

It is well known that TSH stimulates PKA activity via activation of adenylyl cyclase and the production of cAMP. Additionally, elevated levels of TSH are also known to be associated with the development of thyroid cancer in humans (Hargadine et al. 1970, Haymart et al. 2008). However, a mouse model of elevated TSH signaling in a genetically WT background does not develop thyroid cancer (Brewer et al. 2007). The reason behind this discrepancy between mice and humans remains to be determined, and PKA activity has not been directly examined in the previously mentioned model of elevated TSH. In contrast to elevated TSH levels leading to increased PKA signaling, our mice are hyperthyroid, exhibiting low levels of TSH along with activated PKA. These data suggest that TSH signaling may also activate alternate pathways that provide negative feedback on cell growth. This hypothesis may explain why mice with PKA activation develop FTC, while tumors driven by elevated TSH do not develop cancers.

It is interesting to note that ablation of PRKAR1A/Prkar1a from the thyroid is the only genetic change described to date, which produces FTC in both humans and mice. There have been other mouse lines made to generate FTC; however, none of these models mimic both the genetics and phenotypes seen in humans. PTEN mutations have been described in patients with FTC, but the mouse model harboring a deletion of Pten in the thyroid does not develop FTC and instead exhibits thyroid hyperplasia (Yeager et al. 2007). We have also recapitulated this data in our laboratory (data not shown). A small number of FTC patients exhibit mutations in RAS (Niespomniszcze et al. 2006); however, mice expressing the oncogenic allele of Kras in the thyroid show no thyroid abnormalities up to 1 year of age (Miller et al. 2009). In contrast, mice harboring a Pten deletion as well as an oncogenic allele of Kras in the thyroid develop aggressive FTC and lung metastases (Miller et al. 2009), which supports our hypothesis that more than one genetic alteration may be necessary to elicit metastatic thyroid cancer. The mouse expressing a transgene of the PAX8–PPARG fusion protein in the thyroid also fails to develop the FTC seen in patients harboring this genetic translocation (Diallo-Krou et al. 2009). Similar to the Kras mouse model, it has very recently been shown that mice harboring both the PAX8–PPARG fusion protein and Pten deletion in the thyroid develop aggressive and metastatic FTC (Dobson et al. 2011), again indicating that multiple genetic hits are necessary for FTC progression to metastatic disease.

The only other previously published mouse model of FTC, which does develop aggressive FTC, harbors a mutation in Thrb known as the PV mutation (ThrbpPV/PV) (Kato et al. 2004). However, mutations...
in THRb in human patients have, to date, only been seen in patients presenting with thyroid hormone resistance and not FTC (Rocha et al. 2007). Interestingly, ThrbpV/V mice exhibit strikingly high levels of TSH. Thus, the possibility of PKA driving tumor formation in these animals cannot be ruled out. While the effects of TSH stimulation vs the effects of the Thrb mutation on thyrocyte proliferation in this model have been initially examined (Lu et al. 2011), it is still unclear what role the activation of PKA, if any, plays in this model.

Although THRb may not be a tumor suppressor gene in humans, our microarray data support the hypothesis that TRβ may play a role in tumorigenesis in the thyroid. IPA analyses of this data pointed to an altered signaling network involving TRβ (Fig. 3). Interestingly, it has been shown that Spot14 and Ucp1 mRNA and protein as well as Ar levels increase in a hyperthyroid state or after administration of thyroid hormone to hypothyroid mice and rats (Narayan & Towle 1985, Kinlaw et al. 1989, Lee et al. 2007, Martinez de Mena et al. 2010). However, in our animals, the levels of all the three mRNAs and the levels of Spot14 protein dramatically decreased, converse to the expected increases in a hyperthyroid animal. Although the mechanisms of action still require more research, our data indicate that elevated PKA signaling may lead to alteration of TRβ’s normal transcriptional activity, which suggests that the importance of TRβ in FTC development may lie in modulation of its transcriptional activity by other molecules. While these hypotheses require further experimentation, we believe that our data together suggest that PKA has effects on TRβ transcriptional function, which may shed light on how elevated TSH levels lead to FTC formation in humans.

The RAS/RAF/MEK/ERK and PI3K/AKT pathways have long been thought to be major players in the development of FTC (reviewed in Brzezianska & Pastuszak-Lewandoska 2011)); however, our data suggest that this may not be the case, as we have shown FTC formation in the absence of activated Erk or Akt. These data are consistent with our previously published data in both mouse embryonic fibroblasts and Schwann cells, which shows that cellular proliferation in the context of elevated PKA activity is independent of Erk and Akt (Nadella & Kirschner 2005, Jones et al. 2008).

Surprisingly, inhibition of AKT proved to decrease in vitro proliferation of these cells. While this result seems contradictory to our in vivo data, Supplementary Figure 2 shows that once in culture, these cells do show activation of Akt and Erk, indicating an alteration in signaling compared with the in vivo tumors. While these results suggest that this culture system does not perfectly mimic the in vivo tumor conditions, these cells still provide a unique setting for studying the molecular mechanisms of FTC.

In contrast to Akt and Erk, Stat3 was shown to be highly activated in tumors as well as the cultured cells (Fig. 2 and Supplementary Figure 2). Inhibition of Stat3 with HO-3867 did show a significant reduction in cell growth in these cells. Additionally, our quantitative real-time PCR data indicate that this growth inhibition is mediated by effects on Stat3, as treatment of cells with HO-3867 leads to a reduction of mRNA levels of several Stat3 target genes (Fig. 6). While the mRNA levels of Nfil3 were found to be slightly upregulated, this could be due to other regulatory elements on the promoter of this gene as Nfil3 has been shown to be sensitive to levels of insulin and subsequent Akt activation (Tong et al. 2010). As our in vitro data suggest that the tumor cells are dependent on Akt in culture, this may explain the lack of downregulation of Nfil3 upon treatment with HO-3867. While it is impossible to fully rule out off-target effects of this inhibitor, we believe that, together, our data suggest a possible role for Stat3 in the development of FTC in this mouse model.

STAT3 has been implicated in progression and metastases of papillary, anaplastic, and medullary thyroid cancer (Hwang et al. 2003, Trovato et al. 2003, Plaza-Menacho et al. 2007, Kim et al. 2009), but its implications in FTC are not well described. Trovato et al. (2003) described activated STAT3 as only occurring in PTC and not any of a large set of FTC that was examined. To our knowledge, our mouse model is the only report of activated Stat3 in FTC. Recent work has also shown that nuclear Jak2 enhances the stability of activated Creb in the mouse and rat adrenal gland (Lefrancois-Martinez et al. 2011), suggesting that the JAK/STAT pathway may be linked to PKA in endocrine tissues.

Taken together, these data indicate that Prkar1a is indeed a tumor suppressor in the thyroid and that loss of this gene leads to hyperthyroidism and FTC. Our model is the first described model of FTC that is independent of Akt and develops FTC while engineered to harbor a single genetic alteration known to be associated with FTC in humans. In summary, we believe that this mouse is a novel and highly reproducible model of FTC, which may help to elucidate how the TSH/PKA signaling axis contributes to the development of thyroid cancer as well as suggesting new targets, such as STAT3, for the development of FTC therapies.
Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0306.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported in part by NIH grants: CA112268 (L S Kirschner), PO1CA124570 (D R Pringle, L Yu, D Jarjoura, K M D La Perle, and L S Kirschner), and CA16058 (to the OSU Comprehensive Cancer Center). D R Pringle was supported by the Jeffrey J Seilhamer Memorial Fellowship.

Acknowledgements

The authors would like to acknowledge Alan Flechtner, HTL (ASCP) of the Ohio State University College of Veterinary Medicine Comparative Pathology and Mouse Phenotyping Core, for his help with processing, sectioning, and staining tissues for this study; Lisa Rawahneh, Ohio State University, Division of Internal Medicine, for his valuable technical assistance in establishing primary thyroid cell cultures. Additionally, they would like to acknowledge Periannan Kuppusamy, PhD and Brian Rivera of the Davis Heart and Lung Research Institute, The Ohio State University, for providing us with the HO-3867 compound.

References


Niepomnischze H, Suarez H, Pitoia F, Pignatta A, Danilowicz K, Manavela M, Elsner B & Bruno OD 2006 Follicular carcinoma presenting as autonomous functioning thyroid nodule and containing an activating mutation of the TSH receptor (T620I) and a mutation of the Ki-RAS (G12C) genes. *Thyroid* **16** 497–503. (doi:10.1089/thy.2006.16.497)
