The thyroid cancer PAX8–PPARG fusion protein activates Wnt/TCF-responsive cells that have a transformed phenotype

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Abstract

A chromosomal translocation results in the production of a paired box 8–peroxisome proliferator-activated receptor gamma (PAX8–PPARG) fusion protein (PPFP) in ~35% of follicular thyroid carcinomas. To examine the role of PPFP in thyroid oncogenesis, the fusion protein was stably expressed in the non-transformed rat thyroid cell line PCCL3. PPFP conferred on PCCL3 cells the ability to invade through Matrigel and to form colonies in anchorage-independent conditions. PPFP also increased the fraction of cells with Wnt/TCF-responsive green fluorescent protein reporter gene expression. This Wnt/TCF-activated population was enriched for colony-forming and invading cells. These actions of PPFP required a functional PPARG DNA binding domain (DBD) within PPFP and were further stimulated by PPARG agonists. These data indicate that PPFP, through its PPARG DBD, induces Wnt/TCF pathway activation in a subpopulation of cells, and these cells have properties of cellular transformation including increased invasiveness and anchorage-independent growth.

Key Words
- thyroid carcinoma
- PPFP
- Wnt
- TCF

Introduction

The paired box 8–peroxisome proliferator-activated receptor gamma (PAX8–PPARG) fusion protein (PPFP) results from a t(2;3)(q13;p25) chromosomal translocation implicated in the etiology of ~35% of follicular thyroid carcinomas (FTCs; Kroll et al. 2000). The promoter and most of the PAX8 gene are fused to the coding exons of the PPARG gene. As the PAX8 promoter is highly active in the thyroid, PPFP is expressed at a high level in thyroid carcinomas containing this translocation. Expression of PPFP increases cell growth, viability, and anchorage independence in thyroid and non-thyroid cell lines (Powell et al. 2004, Au et al. 2006, Li et al. 2012).

Both fusion partners of PPFP play important roles in tissue development and differentiation. PAX8 is a transcription factor required for thyroid development and mature thyroid gene expression (Esposito et al. 1998, Fabbro et al. 1998, Macchia et al. 1998, Mansouri et al. 1998, Ohno et al. 1999, Magliano et al. 2000). PPARG is a nuclear hormone receptor that has been well studied as the master regulator of adipocyte differentiation and as an
important therapeutic target for diabetes, atherosclerosis, inflammation, and cancer (Tontonoz & Spiegelman 2008). Modulation of PPARG-regulated pathways is thought to be important in PPFP carcinogenesis, and this is consistent with the observation that a different translocation fusing PPARG to the gene CREB3L2 also has been identified in FTC (Lui et al. 2008).

The initial report of PPFP occurrence and subsequent publications demonstrate that PPFP interferes with PPARG transactivation in a dominant negative manner (Kroll et al. 2000, Powell et al. 2004, Yin et al. 2006, 2009). The hypothesis that PPFP exerts its pro-oncogenic effect via repression of PPARG is bolstered by observations that PPARG is downregulated in other types of thyroid carcinoma, that restoration of PPARG activity has anti-proliferative, pro-differentiation effects, and that heterozygous deletion of Pparg enhances tumorigenesis in an unrelated mouse model of thyroid carcinoma (Aldred et al. 2003, Marques et al. 2004, Park et al. 2005, Kato et al. 2006).

Nonetheless, there is evidence that PPFP can also transactivate some Pparg target genes. Numerous Pparg target genes are upregulated in PPFP tumor samples vs non-PPFP FTC or normal thyroid (Lacroix et al. 2005, Giordano et al. 2006). In vitro, PPFP stimulates the promoters of some Pparg target genes, represses others, or both depending on the cellular context (Powell et al. 2004, Au et al. 2006, Giordano et al. 2006).

Thyroid-specific expression of PPFP combined with thyroid-specific knockout of Pten in a transgenic mouse model generates spontaneous metastatic FTC (Dobson et al. 2011). In the thyroids of these mice, Pparg target genes can be positively or negatively regulated by PPFP. With administration of the PPARG agonist pioglitazone, however, adipocyte Pparg target genes are broadly upregulated and thyrocytes adopt an adipocyte phenotype, indicating that the functional domains of PPFP retain their capacity to act in a strongly PPARG-like manner. Thus, overall, the actions of PPFP that contribute to thyroid carcinogenesis are poorly understood.

In this study, we show that expression of PPFP in the rat thyroid PCCL3 cell line induces properties of transformation, including increased anchorage-independent growth and invasiveness. Transformation requires a functional PPARG DNA binding domain (DBD) within PPFP and is further enhanced by PPARG agonists. Our data also show that a small fraction of PCCL3 cells is Wnt/TCF responsive, that the responsive fraction is expanded by PPFP expression, and that this fraction is enriched in cells with the transformed phenotype.

Materials and methods
Cell culture and reagents
The PCCL3-differentiated rat thyroid cell line has been described (Fusco et al. 1987). PCCL3 cells and their derivatives were cultured at 5% CO₂ in Coon’s F-12 media with l-glutamine, 5% fetal bovine serum (FBS), antibiotic/antimycotic (Thermo Scientific, Waltham, MA, USA), 1 mlU/ml thyroid-stimulating hormone, 10 μg/ml insulin, 5 μg/ml apo-transferrin, 10 nM hydrocortisone (Sigma–Aldrich), and prophylactic plasmocin (Invivogen, San Diego, CA, USA).

SR1664 was a gift from Drs Patrick Griffin and Bruce Spiegelman. GW9662 and T0070907 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Bvt.13 was purchased from Sigma–Aldrich. Myc antibody was purchased from Cell Signaling Technology (Danvers, MA, USA, catalog #2276) and GAPDH antibody sc-32233 was from Santa Cruz Biotechnology.

Stable transfection
The P box amino acids EGG within the PPARG DBD of PPFP were mutated to AAA by inverse PCR to generate PPFP-AAA, and the entire sequence was verified. PPFP or PPFP-AAA with three Myc epitopes at the N terminus was inserted into the pCagen plasmid (Addgene, Cambridge, MA, USA, plasmid 11160) (Matsuda & Cepko 2004). pCagen-PPFP, pCagen-PPFP-AAA, or pCagen empty vector (EV) control plasmid was co-transfected at tenfold excess with a hygromycin resistance vector (Clontech, #631625) into PCCL3 cells using Fugene 6 (Promega). Transfected cells were plated at clonogenic density and subjected to hygromycin B selection at 400 μg/ml. Resistant colonies were assayed for PPFP by RT-PCR and western blot.

DNA binding assay
The DNA binding activities of PPFP and PPFP-AAA were assessed using an avidin–biotin complex to DNA assay (Glass et al. 1987) with minor modifications. Whole cell lysates were generated from stably transfected PCCL3 cells using M-PER (Thermo Scientific) supplemented with 0.4 M NaCl and Halt Protease Inhibitor Cocktail (Thermo Scientific). Fifty micrograms of lysate protein were incubated with a double-stranded oligonucleotide, biotinylated at the 5’ end of the top strand, containing the mouse Aqp7 gene PPAR response element (PPRE). The top strand sequence is

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-13-0058
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AGTTCTGTTGCTTTCCAGGGGAGGTCAGTGGCAGGCGGT, and the minimal response element is underlined. A mutated sequence was used as a specificity control in which the underlined sequence was changed to ATTTGAGATTTCA. Protein–DNA complexes were pulled down with NeutrAvidin agarose beads (Thermo Scientific) and were then subjected to electrophoresis through an SDS–PAGE and analyzed for PPFP by western blot using a Myc antibody.

**Lentiviral construction and infection**

All lentivirus construction was done at the University of Michigan Vector Core. Lentiviral TOP destabilized green fluorescent protein (dGFP) construct was a gift of Dr Irving Weissman through Dr Hasan Korkaya. pGreenFire TCF/LEF lentivirus reporter was obtained from System Biosciences (Mountain View, CA, USA). In this construct, dGFP expression is driven by a minimal CMV promoter preceded by four TCF/LEF response elements. The same pGreenFire reporter but lacking TCF binding sites was used as a control to confirm TCF responsiveness. A mutated sequence was used as a specificity control in which the underlined sequence was changed to AGTTCTGTTGTGCTTCTCC. The minimal response element is AGTTCTGTTGTGCTTCTCC.

**Flow cytometry**

Flow cytometric analysis and sorting were performed at the University of Michigan Flow Cytometry Core. Cells to be run on a flow cytometer were filtered through a 40 micron sieve after removal from culture. Cells were resuspended in Hank’s balanced salt solution with propidium iodide (PI) or 4',6-diamidino-2-phenylindole added as a viability indicator. At least 10 000 live cell events were recorded during each run of a cell sample through the flow cytometer. Gates were set at the level of GFP expression in 99.5% of control cells expressing GFP under the control of the minimal CMV promoter without TCF response elements. Test samples with GFP levels higher than gated were considered GFP positive and TCF responsive.

**Soft agar colony formation assay**

Live trypsinized cells in 0.2% trypan blue were counted on a hemocytometer, where at least 95% of cells were verified as singlets. Five thousand cells were resuspended in 333 μl full media, mixed with 667 μl full media plus 0.5% low melt agarose, and added to a well in a 24-well ultra low attachment plate. After the agarose set, 100 μl of full media were added to cover the well. Media were added every 5 days to maintain coverage. After 21 days, cells were stained with 0.025% crystal violet and colonies counted under a microscope.

**Matrigel invasion assay**

Cell culture inserts for 24-well plates from BD Biosciences (Franklin Lakes, NJ, USA) were used with standard tissue culture plates. One hundred microliters of 12.5% Matrigel (BD Biosciences) in unsupplemented F-12 media were added to each insert to cover the base of the top chamber. Matrigel was allowed to set in an incubator at 37 °C for 60 min. Six hundred microliters of Coon’s F-12 media supplemented with 10% FBS were added directly to the bottom well. Five thousand or 25 000 single cells obtained as above were resuspended in 200 μl unsupplemented F-12 media and added to the upper chamber. After 36 h, the experiment was stopped by addition of paraformaldehyde to a final concentration of 1.5%. Afterward, the media were aspirated and a gentle swabbing removed the Matrigel layer and any remaining media. Cells on the bottom of the inserts were stained in a solution of 0.025% crystal violet, washed and stored in distilled water, and counted under a microscope.

**Quantitative PCR and RT-PCR**

Cells removed from culture by trypsinization were placed directly in RealTime Lysis Buffer (Roche Applied Science) and cDNA was synthesized directly from lysate using Transcriptor Universal cDNA Master. Primer and probe sets for the respective genes were ordered from Universal Probe Library (Roche). Quantitative RT-PCR (RT-qPCR) was done on a Lightcycler 480 using Sybr Green I Master. Analysis was done by the LightCycler software at High Sensitivity setting (Roche).

qPCR for PPFP expression was performed as follows. RNA was isolated using a RNeasy mini kit (QIagen). cDNA was synthesized using SuperScript III (Invitrogen). Real-time PCR was performed using an Applied Biosystems Step One Plus real-time PCR instrument and Power SYBR Green master mix. In general, the cDNA from 100 ng RNA was used in triplicate PCR. To analyze lentiviral GFP reporter construct insertion into cell lines, genomic DNA was prepared using a Wizard SV Genomic DNA Purification kit (Promega) and subjected to qPCR. Primer sequences are listed in Table 1.
In vivo studies

All in vivo studies were approved by the University of Michigan Committee on the Use and Care of Animals. FVB/N mice with combined thyroid-specific expression of PPFP and thyroid-specific deletion of Pten (denoted PPFP;PtenFF;Cre) develop FTC that is responsive to pioglitazone, whereas control FVB/N mice with just thyroid-specific Pten deletion (PtenFF;Cre) develop thyroid hyperplasia without neoplasia (Dobson et al. 2011). Thyroid gland RNA from six 10-week-old mice of both genotypes was used to evaluate the expression of potentially TCF-responsive genes by RT-qPCR using the methods described above for PPFP.

Beginning at 8 weeks of age and continuing for 14 days, PPFP;PtenFF;Cre mice were treated with either a control diet, or pioglitazone in the chow at 200 parts per million, or SR1664 by i.p. injection at 30 mg/kg body weight every 12 h. SR1664 at 20 mg/kg is strongly insulin sensitizing (Choi et al. 2011). Thyroid size was measured by ultrasound at the start and end of the 2-week treatment period as described (Dobson et al. 2011).

Statistical analyses

Statistical differences for colony growth, invasion, TCF activation, and gene expression were determined using the Student’s t-test with \( P < 0.05 \) considered significant.

Results

The PPARG DBD within PPFP is essential for the ability of PPFP to induce a transformed cell phenotype

The PCCL3 rat thyroid cell line (Fusco et al. 1987) was stably transfected to express Myc-tagged PPFP or Myc-tagged PPFP with a mutated, non-functional DBD, described in more detail below. Expression of PPFP at the RNA level was 21 ± 2.1-fold above endogenous PPARG by RT-qPCR \((n=3)\), which is within the range of 10- to 50-fold typically seen in PPFP expressing thyroid carcinomas (Giordano et al. 2006).

To determine whether PPFP activities depend on retention of a functional PPARG DBD, we mutated the P box amino acids EGG at the base of the first zinc finger to AAA and stably expressed this mutant PPFP protein in PCCL3 cells. Henceforth, the protein will be denoted as PPFP-AAA and the cell line as PCCL3-AAA. The PPFP and PPFP-AAA proteins were expressed at similar levels in their respective cell lines (Fig. 1A). As expected given that P box amino acids contact the bases in the DNA response element (Umesono & Evans 1989), the AAA mutation prevented binding to a PPRE (Fig. 1B).

Compared with PCCL3 cells stably transfected with EV (PCCL3-EV), PCCL3-PPFP cells exhibited a more transformed phenotype. Thus, PCCL3-PPFP cells generated eight times more colonies in soft agar (Fig. 1C) and three times as many PCCL3-PPFP cells invaded through Matrigel-coated transwells (Fig. 1D). PCCL3-AAA cells did not exhibit increased colony formation or invasion (Fig. 1C and D), indicating that the PPARG DBD within PPFP is important for these activities.

PPFP target genes overlap those from agonist-induced PPARG activation

Gene expression profiles of human PPFP thyroid carcinomas have been reported by Lacroix et al. (2005) and Giordano et al. (2006). Giordano identified 55 genes that were highly overexpressed specifically in PPFP carcinomas,
and 17 of those 55 were also found by Lacroix to be overexpressed in PPFP tumors vs normal thyroids (ACAA1, ALDH1L1, ANGPTL4, AQP7, CHIA, CXCR7 (ACKR3), DHCR24, ENO3, PMP22, FB2N2, FBP1, FGFBP1, MYCL1 (MYCL), PPARG, RAB15, TNFRSF21, and XK). We tested the expression of five of the 17 intersection genes by RT-qPCR and found that three of the five are induced in PCCL3-PPFP cells vs PCCL3-EV cells. The results for these three genes, angiotensin-like 4 (Angptl4), fibroblast growth factor binding protein 1 (Fgfbp1), and myelocytophagocytic viral oncogene homolog 1 (Mycl1), are shown in Fig. 1E. The expression of the other two genes, C-X-C chemokine receptor type 7 (Cxcr7) and tumor necrosis factor receptor superfamily member 21 (Tnfrsf21), was not significantly changed (data not shown). These data indicate that the transcriptional effects of PPFP expression in this rat thyroid cell line overlap with the changes seen in human PPFP carcinomas.

The Connectivity Map (CMAP) is a collection of gene expression data from cultured human cell lines treated with various small molecules, combined with analysis algorithms to facilitate the identification of connections between drugs, diseases, signaling pathways, and gene expression changes (Lamb et al. 2006). We conducted a search on CMAP to identify bioactive small molecules that induce a set of gene expression changes similar to the set common to the Lacroix and Giordano PPFP profiles. The PPARG agonist pioglitazone was among the top results returned (P=0.014 overall, P=0.006 for human prostate PC3 cells) (Table 2). Other thiazolidinediones – rosiglitazone and troglitazone – also elicited significantly
similar responses in PC3 cells \((P<0.05)\) but their overall rankings in CMAP were diminished by large variance in the MCF7 cell responses (data not shown).

**PPFP is a potential modulator of the Wnt pathway**

Another emergent theme from the CMAP results was Wnt pathway modulation. Among the 70 bioactive small molecules eliciting responses significantly enriched for PPFP profile genes, 16 were standard hits in Chembank high-throughput screens for Wnt inhibitors, Wnt/lithium modulators, or \(\beta\)-catenin inducers/translocators (Table 3). Of the 16, simvastatin, azacytidine, methotrexate, amiloride, and anisomycin were also reported to impact on the Wnt pathway elsewhere in the literature (Shim et al. 2004, Lin et al. 2008, Klinghoffer et al. 2009, Qiao et al. 2011, Qiu et al. 2011, Shang et al. 2011, Georgiou et al. 2012, Serafino et al. 2012).

<table>
<thead>
<tr>
<th>Rank</th>
<th>CMAP name</th>
<th>Dose ((\mu M))</th>
<th>Cell</th>
<th>Score</th>
<th>Up</th>
<th>Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>Pioglitazone</td>
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<td>PC3</td>
<td>0.841</td>
<td>0.393</td>
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</tr>
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<td>Pioglitazone</td>
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<td>PC3</td>
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</tr>
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<td>259</td>
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<td>PC3</td>
<td>0.786</td>
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<td>−0.817</td>
</tr>
<tr>
<td>501</td>
<td>Pioglitazone</td>
<td>10</td>
<td>MCF7</td>
<td>0.73</td>
<td>0.308</td>
<td>−0.678</td>
</tr>
<tr>
<td>599</td>
<td>Pioglitazone</td>
<td>10</td>
<td>MCF7</td>
<td>0.713</td>
<td>0.139</td>
<td>−0.824</td>
</tr>
<tr>
<td>648</td>
<td>Pioglitazone</td>
<td>10</td>
<td>PC3</td>
<td>0.705</td>
<td>0.277</td>
<td>−0.676</td>
</tr>
<tr>
<td>1141</td>
<td>Pioglitazone</td>
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<td>PC3</td>
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<td>0.222</td>
<td>−0.623</td>
</tr>
<tr>
<td>2191</td>
<td>Pioglitazone</td>
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<td>MCF7</td>
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<td>0.236</td>
<td>0.598</td>
</tr>
<tr>
<td>2240</td>
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<td>MCF7</td>
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<td>−0.681</td>
</tr>
<tr>
<td>3349</td>
<td>Pioglitazone</td>
<td>10</td>
<td>MCF7</td>
<td>0</td>
<td>−0.144</td>
<td>0.89</td>
</tr>
</tbody>
</table>

**Table 3**  Small molecules that elicited similar gene expression changes to the common PPFP profile, which were also Chembank HTS standard hits for Wnt inhibitor, Wnt/Lithium modulators, or \(\beta\)-catenin inducer/translocator

<table>
<thead>
<tr>
<th>Name</th>
<th>Mean score</th>
<th>(n)</th>
<th>Enrichment</th>
<th>(P)</th>
<th>Specificity</th>
<th>% Non-null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verteporfin</td>
<td>−0.851</td>
<td>3</td>
<td>−0.969</td>
<td>0.00008</td>
<td>0.0058</td>
<td>100</td>
</tr>
<tr>
<td>Colforsin</td>
<td>0.481</td>
<td>5</td>
<td>0.725</td>
<td>0.00364</td>
<td>0.0101</td>
<td>80</td>
</tr>
<tr>
<td>Hymecromone</td>
<td>−0.479</td>
<td>4</td>
<td>−0.749</td>
<td>0.0079</td>
<td>0.0121</td>
<td>75</td>
</tr>
<tr>
<td>Bisacodyl</td>
<td>−0.615</td>
<td>4</td>
<td>−0.748</td>
<td>0.00812</td>
<td>0.0403</td>
<td>75</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>0.6</td>
<td>4</td>
<td>0.742</td>
<td>0.00849</td>
<td>0.0247</td>
<td>75</td>
</tr>
<tr>
<td>Amiloride</td>
<td>−0.603</td>
<td>5</td>
<td>−0.668</td>
<td>0.00953</td>
<td>0.0052</td>
<td>80</td>
</tr>
<tr>
<td>Altizide</td>
<td>−0.315</td>
<td>4</td>
<td>−0.691</td>
<td>0.01971</td>
<td>0.0131</td>
<td>75</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.397</td>
<td>8</td>
<td>0.507</td>
<td>0.02008</td>
<td>0.1024</td>
<td>62</td>
</tr>
<tr>
<td>Azacitidine</td>
<td>0.606</td>
<td>3</td>
<td>0.781</td>
<td>0.02119</td>
<td>0.1394</td>
<td>100</td>
</tr>
<tr>
<td>Securinine</td>
<td>−0.584</td>
<td>4</td>
<td>−0.674</td>
<td>0.02524</td>
<td>0.1456</td>
<td>75</td>
</tr>
<tr>
<td>Proadifen</td>
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<td>−0.671</td>
<td>0.02616</td>
<td>0.0261</td>
<td>75</td>
</tr>
<tr>
<td>Merbromin</td>
<td>−0.345</td>
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<td>−0.605</td>
<td>0.0272</td>
<td>0.1707</td>
<td>80</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>−0.498</td>
<td>4</td>
<td>−0.661</td>
<td>0.02988</td>
<td>0.1864</td>
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<td>Digoxin</td>
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<td>0.03079</td>
<td>0.1122</td>
<td>75</td>
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<td>Lycorine</td>
<td>−0.379</td>
<td>5</td>
<td>−0.581</td>
<td>0.03827</td>
<td>0.28</td>
<td>75</td>
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<tr>
<td>Fluspirilene</td>
<td>0.56</td>
<td>4</td>
<td>0.635</td>
<td>0.04372</td>
<td>0.1005</td>
<td>75</td>
</tr>
</tbody>
</table>

Mean score is the average connectivity score of control-treated pairs for a molecule in the CMAP database, \(n\) indicates the number of such pairs. Enrichment indicates the relative connectivity of a particular set of instances compared with all other instances. Specificity indicates the relative uniqueness of connectivity by tallying the frequency at which the connectivity between the query and relevant instances is equalled or exceeded by the connectivity between the same instances and a large number of gene signatures from MSigDB. Non-null percentage refers to the number of instances that are scored in the same direction as the majority instances.
Based upon this, we delivered via lentivirus two independent Wnt/TCF-responsive promoter constructs driving GFP expression into our cell lines to measure the extent of Wnt pathway activation. GFP-positive cells were sorted and grown to generate new cell lines in which all the cells were transduced. Upon expansion, a substantial fraction of the cells reverted to a TCF_GFP- phenotype. However, with either construct and three independent PPFP cell lines, we found each time a two- to fivefold increase in the number of TCF_GFP+ cells relative to similarly transduced PCCL3-EV control cells. As was the case with the functional assays, the DBD mutant PPFP-AAA did not effect an increase in TCF_GFP activation over control cells (Fig. 2A and B).

An apparent hierarchy exists in the cell lines defined by TCF activation status

We re-sorted the 100% transduced cell lines into TCF_GFP+ and TCF_GFP− fractions. The TCF_GFP+ fraction again generated both TCF_GFP+ and TCF_GFP− populations. The TCF_GFP− fraction also regenerated some TCF_GFP+ cells, but at a small fraction of the original level (Fig. 2C, D, and E). The TCF_GFP− fraction could have had a diminished but extant capacity to generate TCF_GFP− cells, or the TCF_GFP+ cells could have come from contaminating, originally TCF_GFP+ cells. Clonal populations grown from single cells sorted into 96-well plates provided data that support the latter possibility. Of 26 EV control clones and 23 PPFP clones grown from TCF_GFP− cells, only one contained a TCF_GFP+ fraction, which was still a small fraction of the TCF_GFP+ fraction of the parental cell line (Fig. 2F). The ability to reconstitute the heterogeneity of the parental cell lines was clearly confined to the TCF_GFP+ populations of the EV control and PPFP cell lines.

A potential artifactual explanation for the appearance of TCF_GFP− cells could be loss of the TCF/GFP construct in the cellular genome. This was excluded by qPCR measurement of GFP DNA in three PPFP TCF_GFP− and three PPFP TCF_GFP+ clones. Setting the average GFP DNA content of the TCF_GFP+ clones at one, the mean±s.d. GFP content of those clones was 1.00±0.16 and that of the TCF_GFP− clones was 1.07±0.26.

The TCF-responsive cell fraction is enriched for anchorage-independent and invasive cells

Bulk populations of TCF_GFP+ cells formed more colonies in soft agar than TCF_GFP− cells (Fig. 3A). Clones derived from TCF_GFP+ cells also formed more colonies than clones of TCF_GFP− cells, though overlap was observed (Fig. 3B). Furthermore, clones of TCF_GFP+ cells were on average five times more invasive than those of TCF_GFP− cells (Fig. 3C).

A potential artifactual explanation for the lower colony-forming and invasive capacities of the TCF_GFP− cells could be the loss of PPFP expression, which would also result in lower TCF-driven GFP expression. This was excluded by RT-qPCR measurement of PPFP expression in three TCF_GFP− and three TCF_GFP+ clones. The average PPFP expression normalized to β-actin was set at one for the GFP_TCF− cells, resulting in a mean±s.d. PPFP expression of 1.00±0.07 in the TCF_GFP− cells and 1.05±0.08 in the TCF_GFP+ cells.

TCF activation in PCCL3-PPFP cells involves a cell autonomous process

The signaling cascade leading to TCF activation typically originates from exogenous Wnt ligands, but exogenous factors were not sufficient to account for TCF activation in PCCL3-PPFP cells. PCCL3-EV control cells transduced with TCF_GFP+ were co-cultured with fourfold excess PCCL3-EV or PCCL3-PPFP cells constitutively expressing DsRed for 5 days and then analyzed via flow cytometry. After removing DsRed cells from the data, the level of GFP positivity was unchanged (Fig. 3D). PCCL3-PPFP cells co-cultured with fourfold excess control cells also did not lose GFP positivity (data not shown). We conclude that hypothetical activating or inhibiting exogenous ligands are not sufficient for TCF activation, and PPFP activation of TCF pathways occurs via a cell autonomous process.

PPFP also activates Wnt/TCF signaling in vivo

Mice with thyroid-specific expression of PPFP combined with thyroid-specific deletion of the tumor suppressor Pten (denoted PPFP;PtenFF;Cre) develop FTC, whereas mice with just thyroid-specific deletion of Pten (PtenFF;Cre) develop thyroid hyperplasia but no neoplasia (Dobson et al. 2011). We performed gene expression studies of thyroid RNA from mice of these two genotypes to determine whether this in vivo model of PPFP thyroid carcinoma is associated with Wnt/TCF activation. Initially, a comparison was made by Affymetrix microarray analysis. The results of that study will be reported elsewhere, but the GeneChips included 30 genes known to be direct Wnt/TCF targets in other mammalian cell types, 27 of which have been reported to be induced by Wnt/TCF and three repressed (www.stanford.edu/group/nussela/b/cgi-bin/wnt/target_genes).
The microarray analysis indicated that 12 of the 27 potentially inducible genes were induced, and two of the three potentially repressible genes were repressed (data not shown). We selected eight of the induced genes, one repressed gene, and two neutral control genes to evaluate by RT-qPCR in an independent set of six PPFP;PtenFF;Cre mice and six PtenFF;Cre control mice. As shown in Fig. 4, all eight genes expected to be induced were induced, and the gene expected to be repressed was repressed. Thus, PPFP also activates Wnt/TCF signaling in a mouse model of FTC.

Figure 2

(A) Increased TCF-driven GFP expression in PCCL3-PPFP cells. Empty vector (EV) control PCCL3 and PCCL3-PPFP cells were infected with lentiviral constructs carrying a GFP gene driven by a TCF-responsive promoter. Cells that expressed GFP after 5 days in culture were sorted out and expanded to create 100% transfected cell lines. These cell lines were analyzed for GFP expression by flow cytometry. Gates were set at the level of GFP expression in 99.5% of control cells expressing GFP under the control of the same promoter without TCF response elements. Test samples with GFP levels higher than gated were considered GFP positive and TCF responsive.

(B) Increased TCF_GFP positivity in multiple PPFP cell lines vs EV control and AAA cells. EV control PCCL3-EV, PCCL3-PPFP, and PCCL3-AAA cell lines were infected with the TCF reporter construct as described in (A) and analyzed via flow cytometry. Each point on the graph represents an independent cell line. Results are typical of many (> 3) repeated experiments. (C and D) Re-establishment of TCF heterogeneity by TCF_GFP+ cells. The GFP− fractions were placed in culture and analyzed after 5 days. The fractions were analyzed for TCF_GFP expression: (C) a typical GFP− fraction and (D) a typical GFP+ fraction in culture, 5 days after sorting. (E) Cells were sorted according to their GFP expression status and analyzed after 5 days in culture as described in (C and D). A whole population sample for each cell line (gated on cell viability only, denoted PI−) was also sorted through the flow cytometer during the same sorting session and analyzed with the other fractions. Results are means ± S.D. and were repeated with independent cell lines. (F) Re-establishment of TCF heterogeneity in cell lines derived from single TCF_GFP+ cells. Single cells were sorted into each well of a 96-well plate based on their GFP status and grown into cell lines that were analyzed by flow cytometry. Each point on the graph indicates a cell line derived from a single cell, the GFP status of which is indicated below the X-axis. The experiment was repeated and similar results were obtained.
The PPARG agonist pioglitazone increases TCF activation

PCCL3-PPFP cells treated with pioglitazone increased their number of TCF_GFP+ cells by more than twofold (Fig. 5A). Total changes in cell number were insignificant and not a factor in the increased GFP+ percentage (data not shown). Co-treatment with the PPARG antagonist GW9662 negated the effects of pioglitazone, indicating PPARG specificity of pioglitazone action. PCCL3-EV control cells were not affected by the treatment. Cells treated with other PPARG agonists (CAY10410 and GW1929) and another PPARG antagonist (T0070907) yielded similar results (data not shown).

Sorted bulk populations of TCF_GFP– PCCL3-PPFP cells, but not those of PCCL3-EV control cells, saw their TCF_GFP + population increased by pioglitazone to a similar extent as TCF_GFP+ and unsorted populations (Fig. 5B). To address the possibility of contaminating TCF_GFP+ cells being responsible for the re-appearance of TCF_GFP+ cells in the TCF_GFP– population, we also treated clonal populations derived from TCF_GFP– cells. These clones had remained TCF unresponsive, previously containing an average of 0.30% TCF_GFP+ cells. After treatment with pioglitazone, the clones contained an average of 0.90% TCF_GFP+ cells (n=4, P=0.09), suggestive of a small but real induction of TCF_GFP+ cells from the TCF_GFP– population.

The invasiveness of PCCL3-PPFP cells is increased by pioglitazone

Clonal populations grown from individual cells sorted by their TCF status were treated with pioglitazone or DMSO. Compared with the vehicle-treated cells, the pioglitazone-treated cells were more invasive, whether derived from TCF_GFP+ or TCF_GFP– cells (Fig. 5C). The increase was
seen in all but one of the PCCL3-PPFP clones ($n=10$). By contrast, the two PCCL3-EV control clones tested did not become more invasive with pioglitazone.

The effects of full vs selective PPARG agonists on PPFP differ in cell culture vs in vivo

The pioglitazone results suggest that exposure of PPFP carcinomas to this drug would likely increase tumor growth and aggressiveness. However, we previously reported that pioglitazone reduces growth and metastases of tumors in PPFP;PtenFF;Cre mice, a transgenic model of PPFP thyroid carcinoma (Dobson et al. 2011). A remarkable feature of those tumors is that pioglitazone caused them to differentiate toward adipocytes, with the cells accumulating large intracellular lipid droplets and expressing a broad array of PPARG-inducible adipocyte genes.

We hypothesized that the adipogenic response to pioglitazone and other PPARG agonists is separable from the increased TCF responsiveness and invasiveness. This hypothesis is potentially testable due to the existence of selective PPARG modulators (SPPARMs). These small molecules effect the insulin sensitization response of full PPARG agonists with little to no adipogenesis and other associated side effects of classic agonists such as pioglitazone (Doshi et al. 2010).

We found that SPPARMs also increased the activation of TCF. BVT.13 increased the percentage of TCF-responsive cells, and the PPARG antagonist T0070907 blocked this increase (Fig. 5D). Similar results were obtained for FMOC-L-leucine and nTZDpa (data not shown). Each of these reagents was identified as a PPARG-selective agonist with weak adipogenic activity (Rocchi et al. 2001, Östberg et al. 2004, Lacroix et al. 2005, Choi et al. 2010). The maximal effects of the SPPARMs were volatile despite repeated experiments. We present here experiments demonstrating conservative but statistically significant increases in TCF_GFP+ percentage similar to pioglitazone treatment.

SR1664 was recently identified as a selective PPARG agonist with no detectable adipogenic activity (Choi et al. 2011). It also increased TCF_GFP+ positivity in PCCL3-PPFP cells (Fig. 5E). However, PPFP;PtenFF;Cre transgenic mice treated with SR1664 received none of the antitumor effect.
of pioglitazone (Fig. 6), suggesting that the TCF-activating and antitumor effects of PPARG ligands are indeed due to different activities of PPFP.

Taken together, our results suggest the following: i) PPFP’s contribution to malignant transformation depends on its PPARG DNA and ligand binding domains. ii) PPFP increases the number of TCF-responsive PCCL3 cells, a population whose progeny effect the transformed phenotype of PCCL3-PPFP cells. iii) The effects of PPFP are amplified by actions common to PPARG agonists and non-adipogenic selective agonists in vitro. iv) Only the full PPARG agonist has antitumor effects in vivo, suggesting that the associated adipocyte transdifferentiation of cancer thyrocytes is more than a coincidental effect.

Figure 5
(A) Increased TCF activation after pioglitazone treatment. PCCL3-EV and PCCL3-PPFP cells were treated with the PPARG agonist pioglitazone (1 μM) with or without the PPARG antagonist GW9662 (0.1 μM) for 5 days. The cells were then analyzed via flow cytometry for GFP status. (B) Increased TCF activation in each pioglitazone-treated fraction of the PCCL3-PPFP cell line. PCCL3-EV and PCCL3-PPFP cells were sorted via FACS into whole population, TCF_GFP−, and TCF_GFP+ samples. The sorted cells were cultured for 5 days in the presence of pioglitazone (1 μM) or vehicle (DMSO). Cells were then analyzed via flow cytometry for GFP status. (C) Increased invasiveness of pioglitazone-treated PPFP cell lines. Cell lines each derived from a single TCF_GFP− or TCF_GFP+ cell were treated with pioglitazone (1 μM) for 5 days. Cells were then placed in Matrigel-coated transwells and their invasive capacities were analyzed. PPFP expressing cells were more invasive with pioglitazone treatment regardless of whether they were from a GFP− or GFP+ cell. (D) Increased TCF activation by a selective PPARG agonist. Empty vector control and PCCL3-PPFP cells were treated for 5 days with the selective PPARG agonist BVT.13 (1 μM) with or without the PPARG antagonist T0070907 (1 μM). Cells were then recovered and analyzed via flow cytometry for GFP status. (E) Increased TCF activation by non-adipogenic selective PPARG agonist. PCCL3-EV and PCCL3-PPFP cells were treated for 5 days with the non-adipogenic selective PPARG agonist SR1664 (1 μM). Cells were then recovered and analyzed via flow cytometry for GFP status.
Discussion

PPFP was one of the first carcinoma-related translocations to be identified, but the molecular mechanisms underlying its role in thyroid carcinogenesis remain imperfectly understood. PPFP transforms cells in vitro and induces metastatic FTC in mice in conjunction with Pten deletion (Diallo-Krou et al. 2009, Dobson et al. 2011), but it can also inhibit growth in already malignant cell lines and in mouse xenografts of cell lines (Reddi et al. 2011). PPFP stimulates or inhibits targets of PAX8 and PPARG depending on the particular gene and cellular context (Kroll et al. 2000, Powell et al. 2004, Au et al. 2006, Espadinha et al. 2007).

Here, we report that PPFP via its PPARG domains activates the Wnt/TCF pathway and that this is an essential step in the transformation effected by PPFP. Rat thyroid PCCL3 cells expressing PPFP demonstrated higher anchorage independence and invasive potential. These results are consistent with past reports in other cell lines of the pro-oncogenic effects of PPFP (Kroll et al. 2000, Powell et al. 2004, Au et al. 2006, Li et al. 2012). PPFP retains the ability to bind PPREs (Au et al. 2006, Giordano et al. 2006) and our data indicate that a functional PPARG DBD is required for these actions of PPFP.

We reported previously that PPFP is a potent driver of the PPARG transcriptional program in the presence of the classic PPARG agonist pioglitazone (Dobson et al. 2011). We here report that full and selective PPARG agonists enhance the effects of PPFP while PPARG antagonists block them, suggesting an important role for PPARG domains in PPFP biology.

We speculate that the oncogenic targets of PPFP are part of a distinct transcriptional program activated by both full and selective PPARG agonists, rather than the adipogenic program that responds only to full agonists. In vivo in the absence of exogenous ligands, the oncogenic action of PPFP dominates and patients or mice develop FTC, but the presence of a strong full agonist such as pioglitazone allows the adipogenic and antitumor activities of PPFP to prevail.

Based upon CMAP data, we hypothesized that the Wnt/TCF pathway may be a target of PPFP. Wnt/TCF pathways have a central role in cancer and stem cell biology, which is best illustrated by studies in the intestine, where Wnt/TCF activity identifies stem cells that maintain healthy tissues and drive the progression of malignant disease (Korinek et al. 1997, 1998, Barker et al. 2009). Wnt pathway activation is also observed in all forms of thyroid malignancy but is more common and well studied in papillary and anaplastic thyroid carcinomas than FTC (Garcia-Rostan et al. 1999, 2001, Helmbrecht et al. 2001, Zhang et al. 2012).

Expression of PPFP increased the fraction of cells that is Wnt active/TCF responsive, and this fraction is enriched age 8 weeks. Thyroid size was measured by ultrasound at the start and end of treatment. Each line represents one mouse. Thyroid area of WT mice is 1.9 mm² and does not change over the 2-week period (data not shown).
in invasive and colony-forming cells. The TCF-responsive and -unresponsive fractions are hierarchically organized with the former being able to recapitulate the original heterogeneity in TCF reporter activity while the latter remains wholly unresponsive. This hierarchy suggests that the TCF-responsive fraction may harbor PPFP thyroid cancer stem cells, an hypothesis that requires confirmation. Our data suggest that PPFP exerts its oncogenic effects on the small fraction of stem and progenitor-like cells in thyroid cancer, effects that may be obscured in studies where only the bulk population is examined. Our data also suggest that Wnt/TCF activity in PPFP thyroid cancer stem cells may be modulated by small molecules directed at the PPARG domains within PPFP.

Published data on thyroid cancer stem cells are very limited, and no data exist from PPFP carcinomas. However, MET and AKT, both of which can induce TCF/β-catenin-mediated transcription, are reportedly activated in stem cells cultured from undifferentiated thyroid cancers, and this population also has increased nuclear β-catenin (Todaro et al. 2010). Thus, the Wnt/TCF reporter assays we used to identify a functionally significant subpopulation of thyroid cancer cells are promising tools for future use in the study of thyroid stem cells and cancer.

The mechanism of Wnt/TCF pathway activation by PPFP is unclear, except that it requires the PPARG DBD within PPFP. Activation of this pathway is usually mediated by transcription factors of the LEF/TCF7 family. We found that expression of TCF7L2 RNA is induced by PPFP (data not shown). However, shRNA knockdown of TCF7L2 failed to block Wnt/TCF pathway activation (data not shown), indicating that TCF7L2 is either non-essential or not involved in the process. Further studies will be required to understand the mechanism by which PPFP regulates Wnt/TCF signaling.

In conclusion, we identified the Wnt/TCF pathway as a major positively regulated target of PPFP in stably transfected PCCL3 thyroid cells. PPFP activation of the TCF pathway depends on its functional PPARG DNA and ligand binding domains and can be modulated by small molecules in a PPARG-like manner. The Wnt/TCF-activated population is enhanced in the ability to invade through Matrigel and to form colonies in anchorage-independent conditions and may be a novel therapeutic target in patients with PPFP thyroid carcinomas.

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 by NIH grants R01CA151842, R01CA166033, R01CA129765, and T32GM007315 and a Taubman Research Institute award.

Author contribution statement
D Vu-Phan, R J Koenig, and M S Wicha conceived and designed the experiments. D Vu-Phan, V Grachtchouk, J Yu, L A Colby, and R J Koenig performed the experiments. Analyzed the data: D Vu-Phan, V Grachtchouk, J Yu, R J Koenig, M S Wicha. D Vu-Phan, R J Koenig, and M S Wicha wrote the paper.

Acknowledgements
The authors thank Anna Eliassen and Habib Khan for technical assistance.

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Received in final form 31 July 2013
Accepted 12 August 2013