The Putative PAX8/PPAR γ Fusion Oncoprotein Exhibits Partial Tumor Suppressor Activity through Up-Regulation of Micro-RNA-122 and Dominant-Negative PPAR γ Activity

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Abstract

In vitro studies have demonstrated that the PAX8/PPAR γ fusion protein (PPFP), which occurs frequently in follicular thyroid carcinomas (FTC), exhibits oncogenic activity. However, paradoxically, a meta-analysis of extant tumor outcome studies indicates that 68% of FTC-expressing PPFP are minimally invasive compared to only 32% of those lacking PPFP ($\chi^2 = 6.86, P = 0.008$), suggesting that PPFP favorably impacts FTC outcomes. In studies designed to distinguish benign thyroid neoplasms from thyroid carcinomas, the previously identified tumor suppressor miR-122, a major liver micro-RNA (miR) that is decreased in hepatocellular carcinoma, was increased 8.9-fold ($P < 0.05$) in all FTC versus normal, 9.2-fold in FTC versus FA ($P < 0.05$), and 16.8-fold ($P < 0.001$) in FTC + PPFP versus FTC – PPFP. Constitutive expression of PPFP in the FTC-derived cell line WRO (WRO-PPFP) caused a 5-fold increase of miR-122 expression ($P < 0.05$) and a striking 5.1-fold reduction ($P < 0.0001$) in tumor progression compared to WRO-vector cells in a mouse xenograft model. Constitutive expression of either miR-122 or a dominant-negative PPAR γ mutant in WRO cells was less effective than PPFP at inhibiting xenograft tumor progression (1.8-fold [$P < 0.001$] and 1.7-fold [$P < 0.03$], respectively). PPFP-induced up-regulation of miR-122 expression was independent of its known dominant-negative PPAR γ activity. Up-regulation of miR-122 negatively regulates ADAM-17, a known downstream target, in thyroid cells, suggesting an antiangiogenic mechanism in thyroid carcinoma. This latter inference is directly supported by reduced CD-31 expression in WRO xenografts expressing PPFP, miR-122, and DN-PPAR γ . We conclude that, in addition to its apparent oncogenic potential *in vitro*, PPFP exhibits paradoxical tumor suppressor activity *in vivo*, mediated by multiple mechanisms including up-regulation of miR-122 and dominant-negative inhibition of PPAR γ activity.

Keywords

follicular thyroid carcinoma, PAX8/PPAR γ , fusion protein, miR-122, tumor suppressor

Introduction

Follicular thyroid carcinoma (FTC) accounts for up to 20% of all thyroid cancers but is responsible for up to 40% of the deaths associated with this disease.¹ In contrast to the wealth of mechanistic information available regarding the pathogenesis of papillary thyroid carcinoma (PTC), the triggering mechanisms of FTC development have remained obscure. PAX8/PPAR γ rearrangements, which express the PAX8/PPAR γ fusion protein (PPFP), are found in about 36% of FTC.² Because such rearrangements are associated with oncogenesis in other systems,³ PPFP has been implicated in the pathogenesis of FTC. Furthermore, our group and others have confirmed that PPFP acts as an oncogene *in vitro*, with increased rates of cell growth and attachment-independent growth and decreased rates of apoptosis of several cell lines *in vitro*.^{4,5} However, PPFP fails to transform normal thyrocytes in a transgenic animal,⁶ suggesting that other oncogenic events are required to elicit its oncogenic actions. Consequently, the precise role of PPFP in the pathogenesis of FTC remains uncertain.

We have demonstrated that the presence of PPFP in human tumors is associated with reduced CD-31 staining, suggesting decreased neovascularization.² This finding is congruent with our earlier study of a cohort of 54 FTC patients, in which the 31 tumors that expressed PPFP exhibited favorable prognostic indicators, including better tumor

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differentiation and lower metastases rates.⁷ However, there are conflicting data on the prognostic impact of PPFP in FTC, with our group⁷ and several others,⁸⁻¹³ indicating that PPFP is associated with a less aggressive phenotype, while others¹⁴⁻¹⁶ observe a more aggressive phenotype in their patient population. We therefore hypothesize that PPFP acts not as a pure oncogene but as a modulator of oncogenesis, affecting tumor progression in potentially complex ways that ultimately lead to a more favorable outcome.

In studies designed to distinguish benign thyroid neoplasms from thyroid carcinomas, we screened a cohort of FTC with and without PPFP for global micro-RNA (miR) expression. In our preliminary studies, we found that miR-122, a known tumor suppressor,^{17,18} is differentially regulated in FTC compared to normal thyroid or benign/adenomatous tissue and in particular is up-regulated in a subset of FTC-expressing PPFP. miRs are a class of small, noncoding RNAs that negatively regulate gene expression and have been implicated in cancer mechanisms, including both oncogenic and tumor suppressor activities.^{19,20} Analysis of miR expression profiling of a variety of human tumors has identified signatures associated with stage, risk of progression, prognosis, and response to treatment.^{19,20} Currently, only limited information is available on the expression of miRs in FTC,²¹⁻²³ and correlations of miR expression with thyroid disease diagnosis and/or progression have yet to be established. In addition, nothing is known about miR function in thyroid cancer. Consequently, we evaluated PPFP and miR-122 function using an FTC-derived cell line in an *in vivo* xenograft mouse model.

Constitutive expression of PPFP in the follicular thyroid cancer cell line WRO caused up-regulation of miR-122, identifying a novel pathway involved in PPFP function. We demonstrate for the first time that PPFP inhibits FTC tumor progression in nude mouse xenografts through at least 2 independent mechanisms, including dominant-negative inhibition of PPAR γ and up-regulation of miR-122 that negatively impact neovascularization. Our findings illustrate multiple mechanisms are involved in PPFP function in FTC that accounts for its association with less aggressive disease and suggest that PPFP may have potential as a prognostic marker for FTC.

Table 1. Comparison of Differentially Regulated miRNAs among Normal Thyroid, Benign Adenomas, and FTC with and without PPFP

Groups compared	Differentially regulated miRNAs			
	<i>P</i> < 0.05	Mean $\pm$ 1 SD ^a	Mean $\pm$ 2 SD ^a	Mean $\pm$ 3 SD ^a
FTC v. normal	139	74	26	9
FTC v. FA	88	34	5	1
FTC(+PPFP) v. FTC(– PPFP)	130	73	26	8

^aFold change exceeded mean difference by 1, 2, or 3 standard deviations (SDs).

Table 2. Differential Regulation of Selected miRNAs in Normal Thyroids, FA, and FTC with and without PPFP

FTC v. normal (mean $\pm$ 3 SD ^a)		FTC v. FA (mean $\pm$ 2 SD ^a)		FTC(+PPFP) v. FTC(–PPFP) (mean $\pm$ 3 SD ^a)	
miRNA	FC ^b	miRNA	FC ^b	miRNA	FC ^b
hsa-miR-122	8.93	hsa-miR-122	9.24	hsa-miR-122	16.83
hsa-miR-1248	3.34	hsa-miR-221	3.15	hsa-miR-375	4.91
hsa-miR-375	2.77	hsa-miR-375	2.76	hsa-miR-187	3.27
hsa-miR-144*	0.41	HS_29	0.34	hsa-miR-542-3p	0.34
hsa-miR-923	0.31	hsa-miR-642	0.33	hsa-miR-183	0.31
solexa-8048-104	0.29			hsa-miR-339-5p	0.30
hsa-miR-31*	0.28			hsa-miR-450b-5p	0.27
hsa-miR-31	0.26			hsa-miR-542-5p	0.27

^aMean difference was greater than 2 or 3 standard deviations (SDs).

^bFold change (FC).

*Indicates an miRNA expressed at low levels relative to the miRNA in the opposite arm of a hairpin.

Results

Follicular thyroid carcinoma-expressing PPFP is associated with increased miR-122 expression. In studies initially designed to develop markers to distinguish benign thyroid neoplasms from thyroid carcinoma, we profiled a cohort of 10 FA (4 expressing PPFP and 10 without PPFP), 12 FTC (6 each with and without PPFP), and 7 normal thyroid tissues, using miR arrays. There were 139, 88, and 130 miRs, respectively, that distinguished FTC from normal, FTC from FA, and FTC with PPFP from FTC without PPFP (Table 1). A complete listing of the differentially regulated miRs is provided in Supplementary Table S1. Analysis of the expression data demonstrated that miR-122 was significantly up-regulated in FTC (*P* < 0.05), demonstrating a striking increase of 8.93- and 9.24-fold (Table 2) compared to normal thyroid and FA, respectively. More importantly, miR-122 was also able to distinguish a subset of FTC expressing the PAX8/PPAR γ fusion gene (16.83-fold, *P* < 0.001) from FTC that did not (Table 2), suggesting that it

Figure 1. PPFP up-regulates the expression of miR-122 in FTC. Levels of miR-122 were evaluated using qRT-PCR. **(A)** Twelve fresh-frozen FTC, 6 containing PPFP and 6 without PPFP, **(B)** 10 formalin-fixed, paraffin-embedded FTC, 4 with PPFP and 6 without PPFP, **(C)** 7 normal thyroid tissue samples versus 14 FA, and **(D)** a comparison of miR-122 levels in NT-vector and NT-PPFP cells²⁴ *in vitro* versus tumor xenografts *in vivo*. Asterisks indicate statistical significance at the $P < 0.05$ level. Error bars indicate SEM.

may be a PPFP-associated miR. Quantitative PCR (qPCR) of miR-122 expression in the same set of fresh-frozen tumors demonstrated a 128-fold ($P < 0.05$) increase of miR-122 in FTC(+PPFP) versus FTC(-PPFP) (Fig. 1A). These results were also validated in another independent cohort of 10 FTC (6 each without and 4 with PPFP) derived from paraffin-embedded archival tissue that showed a markedly lower but statistically significant 2.28-fold ($P < 0.05$) increase in miR-122 expression in FTC(+PPFP) tumors (Fig. 1B), but not in benign follicular adenomas (Fig. 1C) nor our adenoma model, consisting of immortalized thyrocytes (Nthy-ori 3-1 [NT] cells) constitutively expressing PPFP² (Fig. 1D). These data confirm the miR profiling results and demonstrate that PPFP expression in follicular carcinomas, but not adenomas, is associated with significant up-regulation of miR-122.

Constitutive expression of miR-122 in the FTC-derived cell line WRO results in reduced tumor progression in a xenograft mouse model. Because miR-122 exhibits tumor suppressor function in liver cells,^{17,18} we examined whether it also serves a tumor suppressor function in thyroid cells. We generated a stable cell line expressing miR-122 in the PPFP-negative FTC-derived cell line WRO, which resulted in an

Figure 2. Overexpression of miR-122 in FTC-derived WRO cells inhibits tumor progression in nude mouse xenografts. **(A)** Stable transfection of a miR-122 precursor in WRO cells resulted in an approximate 8,000-fold increase ($P < 0.005$) in miR-122 expression as evaluated by quantitative RT-PCR. Expression of miR-122 in WRO cells results in a modest but significant (11%) increase in cell growth **(B)** and a significant inhibition (~2-fold) of tumor progression as assessed by repeated-measures (RM) ANOVA ($P = 0.0082$ and 0.0011 , respectively). The values in **B** represent mean $\pm$ SD from 3 independent experiments, each done in triplicate, and values in **C** represent mean $\pm$ SEM from 16 and 10 independent tumors each for WRO-miR-null and WRO-miR-122 cells, respectively. Asterisks indicate statistical significance at the $P < 0.05$ level.

8,000-fold increase in miR-122 levels versus miR-null vector-expressing cells (Fig. 2A). Overexpression of miR-122 in WRO cells increased *in vitro* growth slightly but significantly (11% increase at 72 hours, $P < 0.0082$) (Fig. 2B). However, when these cells were transplanted into nude mice, tumor progression was inhibited by 1.8-fold ($P < 0.0011$) (Fig. 2C), demonstrating that miR-122 can exert tumor suppressor activity in FTC-derived cells. This raises the question whether PPFP itself might also exhibit tumor suppressor function.

Stable expression of PPFP in WRO cells up-regulates miR-122 expression and inhibits cell growth *in vitro* and xenograft tumor progression *in vivo*. To understand the functional consequence of PPFP's association with increased miR-122 expression in FTC, we constitutively expressed PPFP under the control of a CMV promoter (Fig. 3A) in the FTC-derived WRO cell line. Expression of PPFP was verified using RT-PCR (Fig. 3B). Expression of PPFP in WRO cells resulted in a 19% decrease ($P < 0.0001$) in cell growth *in vitro* compared to WRO-vector cells (Fig. 3C) and resulted in a 5.1-fold inhibition ($P < 0.0001$) of xenograft tumor progression at 5 weeks (Fig. 3D). Quantitation of miR-122 levels demonstrated an 8- and 40-fold increase ($P < 0.05$) in the WRO-PPFP cells and xenografts, respectively (Fig. 3E), confirming that this WRO model recapitulates the properties seen in PPFP-expressing human FTC (Table 3).

Dominant-negative inhibition of PPAR is not involved in PPFP-mediated up-regulation of miR-122. Since our earlier observations demonstrated that PPFP functions at least in part by dominant-negative inhibition of PPAR γ ,^{4,24} we generated stable cells expressing a dominant-negative PPAR γ mutant (DN-PPAR γ)²⁴ in WRO cells (Fig. 4A and 4B). Reporter assays confirmed that activation of the PPAR γ response element (PPRE) was significantly inhibited in both WRO-PPFP (29%) and WRO-DN-PPAR γ (44%) cells (Fig. 4C), demonstrating that PPFP retains its dominant-negative PPAR γ activity in the WRO-PPFP cells and that the DN-PPAR γ mutant is expressed in a functional manner in WRO-DN-PPAR γ cells. Expression of the DN-PPAR γ mutant in WRO cells inhibited *in vitro* cell growth by 29% at 72 hours ($P < 0.0001$) (Fig. 4D). While this inhibition appeared to be somewhat greater than that of PPFP (19%) (Fig. 3C), there was no significant difference as assessed by repeated-measures ANOVA. Despite their similar ability to inhibit *in vitro* cell growth, DN-PPAR γ was much less effective than PPFP in inhibiting xenograft tumorigenesis *in vivo* since it exhibited modest inhibition (1.7-fold, $P < 0.0074$) (Fig. 4E). Further, miR-122 levels were not significantly increased in the WRO-DN-PPAR γ mutant cells (Fig. 4F), indicating that up-regulation of miR-122 by PPFP was

Figure 3. Stable expression of PPFP in FTC-derived WRO cells up-regulates miR-122 expression and inhibits xenograft tumor progression. The FTC-derived cell line WRO was genetically engineered to stably express PPFP using the vector shown in **A**. RT-PCR of WRO-PPFP cells (**B**) verifies expression of PPFP. (**C**) Expression of PPFP resulted in a 19% ($P < 0.0001$) inhibition of cell growth *in vitro*. (**D**) Subcutaneous injection of 5×10^6 cells of WRO-PPFP cells into the right and left flanks of athymic nude mice was monitored over a period of 6 weeks and tumor volume measured and plotted as a function of time. Expression of PPFP in WRO cells results in significant inhibition (5.3-fold) of tumor progression as assessed by repeated-measures (RM) ANOVA ($P = 0.0001$). (**E**) Quantitation of miR-122 expression by RT-PCR demonstrated a significant increase ($P < 0.05$) in the WRO-PPFP cells and xenograft tumors versus the vector control. The values in **C** represent mean $\pm$ SD from 3 independent experiments, each done in triplicate. Values in **D** and **E** represent mean $\pm$ SEM from 8 independent tumors each for WRO-vector and WRO-PPFP cells. Asterisks indicate statistical significance at the $P < 0.05$ level.

Table 3. Relative miR-122 Expression (Fold Difference) in WRO Models Expressing PPFP and FTC Expressing PPFP Compared to WRO Control Cells and FTC without PPFP

WRO models expressing PPFP		
Cell lines	Xenografts	FTC (+PPFP)
8.0 ($P < 0.05$)	40.0 ($P < 0.05$)	16.8 ($P < 0.001$)

not mediated by its dominant-negative inhibition of PPAR γ activity. Treatment of WRO-vector cells with GW9662, a potent antagonist of PPAR γ , confirmed these observations since there was a 38% ($P < 0.05$) decrease in PPRE activity (Fig. 4G) that was not accompanied by increased miR-122 expression (Fig. 4H). Taken together, these data indicate that PPFP-associated up-regulation of miR-122 is not mediated by its dominant-negative PPAR γ inhibitory function. Thus, PPFP appears to be a more potent tumor suppressor in WRO cells than either miR-122 or DN-PPAR γ alone, presumably acting through multiple pathways.

Figure 4. PPFP functions in part by dominant-negative inhibition of PPAR γ . **(A)** Scheme for generating the DN-PPAR γ expression construct. **(B)** Expression of PPAR γ (top panel), DN-PPAR γ (middle panel), and β -actin (bottom panel) proteins as demonstrated by Western blotting of WRO-vector, -PPFP, and -DN-PPAR γ cell extracts. **(C)** WRO-vector, -PPFP, or -DN-PPAR γ cells were transiently transfected with the PPRE-Luc reporter and assayed for PPAR γ activity, which is expressed as a percentage of WRO-vector-transfected cells. **(D)** The DN-PPAR γ mutant resulted in a 29% ($P < 0.0001$) inhibition of cell growth *in vitro*. **(E)** Subcutaneous injection of 5×10^6 cells of WRO-vector, -PPFP, or -DN-PPAR γ cells into the right and left flanks of athymic nude mice was monitored over a period of 6 weeks and tumor volume measured. Expression of DN-PPAR γ in WRO cells results in significant inhibition (1.8-fold) of tumor progression as assessed by repeated-measures (RM) ANOVA ($P = 0.0074$) but was not as effective as PPFP. miR-122 expression was not up-regulated when PPAR γ activity was inhibited either with a DN-PPAR γ mutant (**F**) or when WRO-vector cells were treated with GW9662, a potent PPAR γ antagonist (**H**), which significantly inhibited PPAR γ (PPRE) activity (**G**). Data in **C**, **D**, and **F** through **H** represent a mean of 3 experiments performed in triplicate. Data in **E** represent a mean of 8, 11, and 16 tumors each for vector, PPFP, and DN-PPAR γ cells, respectively. Asterisks indicate statistical significance at the $P < 0.05$ level. Error bars indicate SEM.

Up-regulation of miR-122 in WRO cells reduces expression of the proangiogenic factor ADAM-17, implicating antiangiogenesis in the tumor suppressor activity of PPFP. To ascertain whether PPFP-mediated up-regulation of miR-122 had functional consequences in our model systems, we evaluated transcript levels of ADAM-17, a known downstream target of miR-122.¹⁸ Figure 5A demonstrates that reduction of ADAM-17 mRNA expression is observed only in cell lines that display significant up-regulation of miR-122, including the WRO-miR-122 cells (100% reduction, $P < 0.0001$) and WRO-PPFP cells (50% reduction, $P < 0.001$), but not immortalized thyrocytes (Nthy-ori cells) expressing PPFP.²⁴ Evaluation of ADAM-17 expression in human tumors also demonstrated that ADAM-17 was

reduced 20-fold ($P < 0.05$) only in the follicular carcinomas that express PPFP (Fig. 5B), but not in the adenomas (Fig. 5C). These data indicate that PPFP-mediated up-regulation of miR-122 in our cell lines and human tumors exerts functional consequences on miR-122 downstream targets and suggest that PPFP-mediated miR-122 up-regulation may act as a tumor growth modulator by inhibiting angiogenic pathways.

PPFP-mediated inhibition of neovascularization partially accounts for its tumor suppressor function. The xenograft mouse model experiments clearly demonstrate that PPFP is a more potent inhibitor of tumor progression compared to either DN-PPAR γ or miR-122 (Figs. 2-4). Given our previous

Figure 5. ADAM-17 is down-regulated in the presence of miR-122. Quantitation of ADAM-17 transcript demonstrates a significant down-regulation in the presence of miR-122, which is restricted to the WRO-miR-122 ($P < 0.0005$) and WRO-PPFP ($P < 0.005$) cells (A) and FTC tumors containing PPFP (B). Normal thyroid tissues and follicular adenomas (FA) do not show reduction in ADAM-17 expression (C). Asterisks indicate statistical significance at the $P < 0.05$ level.

observations that PPFP expression in human adenomas and carcinomas is associated with decreased neovascularization,²⁴ the WRO-vector, -PPFP, -DN-PPAR γ , and -miR-122 xenografts were evaluated for microvessel density (MVD) as evidenced by CD-31 staining (Fig. 6A). Quantitation of CD-31 staining demonstrated that MVD was significantly decreased 2.1-, 1.7-, and 3.4-fold, respectively, in the WRO-PPFP, -DN-PPAR γ , and -miR-122 xenografts compared to control xenografts, but they were not significantly different from each other (Fig. 6B). The data therefore suggest that PPFP's more potent tumor suppressor function may be mediated by mechanisms other than inhibition of neovascularization alone.

Figure 6. Neovascularization of WRO xenografts is inhibited by PPFP, DN-PPAR γ , and miR-122 expression. (A) Immunohistochemical staining of WRO-vector, -PPFP, -DN-PPAR γ , and -miR-122 xenograft tumors by CD-31 for quantitation of microvessel density (B) demonstrated that neovascularization is decreased 2.1-, 1.7-, and 3.4-fold in PPFP, miR-122, and DN-PPAR γ xenograft tumors, respectively ($P < 0.05$), compared to WRO-vector xenografts. Asterisks indicate statistical significance at the $P < 0.05$ level. Error bars indicate SEM.

Discussion

The mechanisms involved in follicular thyroid cancer development and progression are not well understood. Although genetic alterations such as RAS and PPAR γ rearrangements, including PAX8/PPAR γ ²⁵ and CREB3L2/PPAR γ ,²⁶ have been identified in subsets of FTC, their pathogenic role remains unknown. Several *in vitro* studies employing transient expression of PPFP in various thyroid-derived cell lines have suggested that PPFP has oncogenic functions, including the ability to promote cell growth, inhibit apoptosis, and enhance anchorage-independent growth and migration.² However, support for an oncogenic role for PPFP has not been forthcoming from the extant *in vivo* studies. Although PPFP initially accelerates tumor growth after injection of PPFP-transformed immortalized thyrocytes (Nthy-ori 3-1 cells) into nude mice, the tumors regress spontaneously and are seen to exhibit decreased vascularization and angiogenesis.² Two PPFP transgenic models have been developed, one targeting PPFP to mammary epithelial cells²⁷ and the other targeting it to the thyroid.⁶ In neither case did PPFP induce carcinoma. PPFP did inhibit mammary epithelial cell PPAR γ activity, increasing these cells' susceptibility to carcinogen-induced tumorigenesis.²⁷ In the thyroid-specific transgenic mouse model, a small proportion (25%) of mice expressing PPFP exhibited mild benign hyperplasia, which appeared to be enhanced in the presence of PTEN haploinsufficiency.⁶ These studies suggest that PPFP is not a primary oncogene, capable of cell transformation, but do not exclude the possibility that it

Table 4. Meta-analysis of Published Association Studies Defining the Value of PPFP as a Prognostic Marker

Study	Total	FTC		-PPFP		+PPFP		Reference
		-PPFP	+PPFP	MI	WI	MI	WI	
1	15	7	8	6	1	4	4	(34)
2	9	4	5	4	0	5	0	(8)
3	28	16	12	7	9	1	11	(14)
4	42	31	11	19	12	2	9	(15)
5	17	11	6	7	4	5	1	(9)
6	34	24	10	20	4	8	2	(10)
7	21	17	4	5	12	1	3	(16)
8	34	16	18	10	6	16	2	(11)
9	54	23	31	0	23	28	3	(7)
10	27 ^a	12	10	7	5	6	4	(35)
11	17	10	7	4	6	7	0	(12)
12	12 ^b	8	4	ND	ND	4	0	(13)
Total	276	171	122	89	82	83	39	
Percentage total				50.5	46.5	68 ^c	32	

^aPPFP status was not determined in 5 tumor samples, and these were excluded from this analysis; all were classified as minimally invasive.

^bBreakdown of sample pathology was not determined (ND) for the tumors lacking PPFP. Consequently, this entire data set was excluded from the analysis.

^cUncorrected $\chi^2 = 7.51$, $P = 0.006$, and Yates-corrected $\chi^2 = 6.86$, $P = 0.008$.

may play a secondary oncogenic role in the context of other oncogenic events.

We previously reported that PPFP expression is associated with tumors that exhibit a mildly invasive phenotype and reduced metastatic potential based on a retrospective study of 54 FTC, 31 of which expressed PPFP.⁷ This association is supported by a meta-analysis of all FTC outcomes data reported in the literature (Table 4), which indicates that 68% of FTC-expressing PPFP are minimally invasive ($\chi^2 = 6.86$, $P = 0.008$), suggesting that most PPFP-expressing tumors are more likely to be minimally invasive than tumors that fail to express this fusion protein. Recently, we showed that PPFP expression in FTC is also associated with decreased vascularization.² These data are consistent with PPFP exerting a paradoxical tumor suppressor function, which may be mediated in part through antiangiogenic activity.

Using miR profiling, we identified and verified a specific miR, miR-122, that is up-regulated and differentiates a subset of FTC containing the PAX8/PPAR γ rearrangement. Since miR-122 has been shown to be a tumor suppressor in liver cells^{17,18} and is reduced in hepatocellular carcinoma (HCC),²⁸ we decided to explore its function in thyroid cells. Using an FTC-derived cell line (WRO), we have generated biological models constitutively expressing PPFP, miR-122, and DN-PPAR γ and have demonstrated *in vivo*, using nude mice xenografts, that PPFP has a dual effect on inhibition of tumor growth through at least 2 independent mechanisms: up-regulation of miR-122 expression and dominant-negative inhibition of PPAR γ .

The finding that miR-122 up-regulation specifically distinguishes a subset of FTC containing the PAX8/PPAR γ translocation (Table 3 and Fig. 1) underscores the potential role of PPFP as a tumor modulator with tumor suppressor activity. MiR-122 was first recognized as an abundant liver-specific MiR, which is significantly down-regulated in HCC²⁸ and has been directly associated with tumor suppressor function, mediated through multiple mechanisms, including antiangiogenic activity^{17,18} and its ability to inhibit metastasis in HCC.¹⁸ Accordingly, PPFP-mediated up-regulation of miR-122 appeared to provide at least one mechanism by which PPFP could inhibit tumor progression in mouse xenografts, and this was confirmed by showing that FTC-derived cells that constitutively express miR-122 exhibit delayed tumorigenesis in the nude mice xenograft studies (Fig. 2). Interestingly,

the up-regulation of miR-122 was considerably higher (40-fold) in the tumor xenografts than in the original cells (8-fold) (Fig. 3E), suggesting that the cellular microenvironment also influences its expression.

Several putative targets have been identified for miR-122, including vimentin, ADAM-10, SRF, IGF-1,¹⁷ and ADAM-17.^{17,18} However, the mechanism(s) by which miR-122 expression is regulated is not known. PPFP has been thought to function primarily by dominant-negative inhibition of PPAR γ activity^{4,25}; however, expression of a DN-PPAR γ mutant or use of a potent PPAR γ antagonist did not result in similar up-regulation of miR-122 in WRO cells (Fig. 4), indicating that PPFP-mediated up-regulation of miR-122 occurs through a mechanism independent of its DN-PPAR γ activity. The associated decrease of ADAM-17 (Fig. 5), a downstream target of miR-122,¹⁸ validated these observations, confirmed the functionality of miR-122, and suggested that regulation of ADAM-17 may play a role in the amelioration of tumor progression in PPFP-expressing FTC. This latter conclusion was supported by direct measurement of CD-31 to document the inhibition of vessel formation in the xenograft tumors (Fig. 6). While these data demonstrate reduced neovascularization in tumors, it is important to point out that inhibition of neovascularization is unlikely to explain all of the tumor suppressor function of miR-122, PPFP, and DN-PPAR γ . This follows because these 3 agents differentially affect tumor suppression, with PPFP exhibiting much greater activity than either miR-122 or DN-PPAR γ ; yet, all 3 agents inhibit CD-31 staining to a

similar degree. Thus, additional mechanisms are likely to play a role in tumor suppression.

One possible mechanism for the enhanced tumor suppressor activity of PPFP relative to dominant-negative inhibition of PPAR γ and up-regulation of miR-122 alone may be that these pathways may act additively or synergistically. PPFP may also affect other pathways that exert a tumor suppressor role. The impact on cancer development and progression of PPAR γ itself is controversial, being associated with both oncogenic and tumor suppressor functions: Activation of PPAR γ inhibits the proliferation of malignant cells from various lineages,²⁹ and the presence of PPAR γ somatic mutations in certain colon cancers supports the concept that PPAR γ functions as a tumor suppressor. However, PPAR γ activation is also associated with the development of colon tumors.²⁹ In the case of thyroid cancer, several studies have demonstrated that PPAR γ insufficiency plays a role in FTC development and progression in a mouse model of FTC,^{30,31} although the relevance of this model to human FTC is unknown. Given the multiple roles of PPAR γ , it is possible that it sits at a critical nexus affecting both oncogenic and tumor suppressor pathways, which may explain some of the paradoxical functions of PPFP, which acts at least in part through inhibition of PPAR γ pathways.

In summary, our data indicate that PPFP, like PPAR γ , exhibits both oncogenic potential (at least *in vitro*) and tumor suppressor function *in vivo*. The presence of PPFP in FTC may favorably impact tumorigenesis by delaying tumor progression, and it may act as a favorable prognostic indicator. Our data provide further understanding of the mechanism of PPFP action *in vivo*, including independent tumor suppression actions, through dominant-negative PPAR γ inhibition and up-regulation of the tumor suppressor miR-122.

Materials and Methods

Generation of stable cells. WRO cells (a gift from Dr. Bryan Haugen, University of Colorado, Denver, CO) were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) and 1x penicillin-streptomycin (Invitrogen) at 37°C in humidified conditions with 5% CO₂. PPFP and dominant-negative PPAR γ constructs were generated previously.²⁴ To generate stable lines, cells were seeded at 1×10^6 cells per 100-mm petri dish and transfected 24 hours later in multiples of 3, with 10 μ g each of a vector or expression cassette (PPFP or DN-PPAR γ) using LT-1 (Mirus Bio, Madison, WI). For the generation of miR stable cells, mmu-null and mmu-miR-122-EGFP constructs (Cell Biolabs, San Diego, CA) were used. After 72 hours, WRO-vector, WRO-PPFP, and WRO-DN-PPAR γ cells were obtained by selection in

media containing 400 μ g/mL of G418 (Invitrogen). WRO-miR-null and WRO-miR-122 cells were generated using puromycin (Invitrogen) selection (2.5 μ g/mL). Pools of cells were harvested and analyzed for mRNA and miR expression by quantitative RT-PCR (qPCR) and protein by Western blotting.

RNA extraction and RT-PCR. Total RNA from each group of cells was isolated using TriZol Reagent (Invitrogen). RNA (1 μ g) was reverse transcribed with oligo-dT primers using the Message Sensor RT kit (Ambion, Austin, TX). PAX8/PPAR γ fusion gene was detected by PCR using 5 μ L of cDNA and a sense primer from exon 7 of PAX8, 5'-CGC GGATCCGCATTGACTCACAGAGCA-3' and antisense primer from exon 1 of PPAR γ , 5'-CCGGAATTCTGAAGT CAACAGTAGTGAA-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified using kit primers. ADAM-17 transcript was detected as per Tsai *et al.*¹⁸ At least 2 xenograft tumors per group were harvested at the end of the study and homogenized in T-PER (Pierce, Rockford, IL), RNA extracted, and RT-PCR performed for PPFP and GAPDH as described above. Annealing temperature for PPFP, ADAM-17, and GAPDH was 55°C for 30 seconds. miR-122 status was verified using TaqMan microRNA assays (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions.

To determine ADAM-17 expression in the different models relative to miR-122 levels, ADAM-17 and miR-122 were independently quantitated and normalized to their internal controls, GAPDH and miR-16, respectively. Each model was calibrated to its respective control and a ratio obtained between ADAM-17 and miR-122 for each model.

Protein expression analysis. Stably transfected cells were harvested 24 hours postseeding in M-PER reagent (Pierce), and protein was quantified (Coomassie, Pierce). Protein expression was assayed by Western blotting using the extended West Dura substrate (Pierce). PPAR γ , DN-PPAR γ , and actin were detected with H-100 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Flag M2 (Stratagene, La Jolla, CA), and anti-human β -actin (Sigma-Aldrich, St. Louis, MO) antibodies, respectively. Detection and densitometric analysis were performed with the Kodak ISO 4000 instrument (Rochester, NY) with normalization to β -actin.

Luciferase reporter assays. Cells (0.3×10^6 cells/well) in 6-well plates were transfected using LT-1 reagent (Mirus Bio) with 10 ng of Renilla (Promega, Madison, WI) and 100 ng of PPFP-Aco-Luc reporter²⁵ in triplicate. After 24 hours, cells were lysed and assayed using the dual luciferase kit (Promega). Data were calculated as a ratio of the luciferase to Renilla expression and plotted as fold change versus vector. To test the effect of GW9662 on PPAR γ

activity, 18 hours posttransfection, cells were treated either with 10 μ M GW9662 or an appropriate concentration of DMSO vehicle for 24 hours prior to harvest and assay.

Micro-RNA arrays. Global miRNA expression was evaluated in 27 fresh-frozen thyroid tissues including 8 FA (1 with and 7 without PFP), 12 FTC (6 with and 6 without PFP), and 7 normal samples using the human v2 miR panel from Illumina at the Mayo Clinic Microarray Shared Resource. Ratios were generated between different groups; fold changes were calculated, sorted with mean expression differences ≥ 1 , 2, or 3 SD, and then analyzed in independent groups. The expression of miR-122 was confirmed in the same set of tumors using qPCR as described above and also validated in another independent cohort of 10 formalin-fixed paraffin-embedded (FFPE) tissues, including 10 FTC (4 with and 6 without PFP), 6 FA (3 with and 3 without PFP), and 3 normal samples. RNA from fresh-frozen tissues and FFPE blocks was extracted using MiRVana and Recover All (Ambion) kits, respectively, per manufacturer protocols.

In vitro cell growth assay. Cells were plated (5×10^5 per plate) in 12-well plates. After 24, 48, and 72 hours, cell viability was quantified by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrasodium bromide, Sigma-Aldrich) per Denizot *et al.*³²

In vivo xenograft tumor formation assays. Stable cells (5×10^6) were resuspended in 100 μ L of growth media and injected subcutaneously into both flanks of 4-week-old athymic nude mice (Fox1^{nu/nu}, Harlan, Indianapolis, IN). At least 3 mice (6 tumors) were injected per group. Tumor size was determined weekly by measuring tumor length (L), width (W), and height (H) using vernier calipers ($V_{\text{tumor}} = 0.5236LWH$). Fold differences in tumor progression between the control and experimental groups were measured as the ratio of the areas under the respective curves. Animal studies were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Tumor histology, immunohistochemistry, and pathological evaluation. At least 4 tumors per group were formalin fixed and paraffin embedded and individual sections stained with CD-31 (1:200 dilution, sc-1506-R, Santa Cruz Biotechnology) for 30 to 60 minutes and counterstained with hematoxylin. Antigen retrieval was done in EDTA at 37°C for 30 minutes after deparaffinization. Sections were blocked with peroxidase and protein for 5 minutes, respectively. Antibody staining was visualized using Dako Envision plus System (Glostrup, Denmark) for 15 minutes followed by DAB chromogen for 10 minutes.

Stained slides were scanned using the Hamamatsu Nano-Zoomer (Bacus Laboratories Inc., Chicago, IL). Digital

images composed of multiple tiles (4096×64 pixels) were captured at 20x magnification. Image analysis was conducted according to Monahan *et al.*³³ For each tumor section, the relative signal intensity from 10 randomly chosen fields (205×300 pixels) was quantified by histogram analysis (Adobe Photoshop, San Jose, CA). Data were expressed as signal intensity (signal pixel count divided by total image pixel count).

Statistics. The statistical significance of difference between control and PFP groups was determined by the Student 2-tailed *t* test, and $P < 0.05$ was considered significant. For experiments where multiple comparisons were being examined, the data were subjected to ANOVA with a *post hoc* Bonferroni test. Tumor progression and *in vitro* cell growth were assessed by repeated-measures ANOVA. All analyses were performed utilizing SAS 9.2 software (Cary, NC).

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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