SUPPLEMENTARY MATERIALS

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Patient specimens. Ethics approval was obtained through the University of Alberta Heath Research Ethics Board ID Pro00018758. Specimens prepared for primary cell culture or tissue banking were placed in culture media or OCT (Optimal Cutting Temperature compound), respectively, within 15 minutes of devascularisation. For the tissue array with paraffin specimens, a total of 287 patient specimens were selected with thyroid tumors of which 181 are papillary thyroid carcinomas (113 without and 68 with lymphatic metastases), 57 are benign follicular neoplasms and there are 36 normal thyroid tissue specimens and 13 section of metastatic lymph nodes. In all cases of suspicious or proven malignant disease patients typically had a total thyroidectomy with consideration of a level VI lymph node dissection. Two pathologists separately assessed the specimens to document primary tissue diagnosis as well as the presence of lymphatic metastases in nodes sectioned. Clinical data was obtained from a prospective database maintained at the Cross Cancer Institute tracking disease from 2002 onwards. All of the patients are followed from a single site at the multidisciplinary thyroid tumor board. Recurrence is defined as an increase in unstimulated thyroglobulin levels of greater than 0.4 ng/ml, stimulated thyroglobulin levels greater than 2 ng/ml, and/or pathologic evidence of recurrence based on ultrasound-guided fine needle aspiration biopsy. Dosimetry records obtained directly from the radioisotope records maintained at the Cross Cancer Institute.

Reagents and Antibodies. Doxycycline hyclate was purchased from Sigma-Aldrich (St Louis, MO, USA). Platelet-derived growth factor (PDGF-AA and-BB) were from GIBCO (Invitrogen, USA). Crenolanib was purchased from Selleckchem (Houston, TX, USA) and used at 0.8mg/mL. Propidium iodide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Okadaic acid and Sodium orthovanadate were also from Sigma-Aldrich and used at 1μM and 200μM respectively. The following antibodies were used for immunoblotting, immunofluorescence and for staining the paraffin tissue arrays: PDGFRα (D13C6: #5241), phospho-PDGFRα/β (Tyr849)/(Tyr857) (C43E9: #3170), phospho-TTF1 (Ser327) (#13608) were from Cell Signaling Technology (Danvers, MA, USA). The PDGFRβ (11H4:sc-80991), Pax8 (PAX8R1:sc-81353), TTF1 (8G7G3/1:sc-53136) and GAPDH (FL-335:sc-25778) antibodies were from Santa Cruz Biotechnology, (Dallas, TX, USA). Anti-γ-tubulin antibody (GTU-88:T6557) was from Sigma-Aldrich (St. Louis, MO, USA). For cell sorting of PDGFRα positive cells we used PDGFRα/PE conjugate (D13C6:#8533) from Cell Signaling Technology (Danvers, MA, USA). Goat anti-Mouse IgG (H+L) Alexa Fluor® 488 conjugate (A-11029) and chicken anti-Rabbit Alexa Fluor® 594 conjugate (A-21442) secondary antibodies were from Life Technologies (Waltham, MA, USA).

Cell culture. FRTL5, SW579 and BHT101 cells were from ATCC (Manassas, VA, USA), BCPAP and 8305C cells were purchased from DSMZ (Braunschweig, Germany) where cell lines were authenticated by short tandem repeat analysis. TPC1 and KTC-1cell lines were generously provided by Dr. S. Ezzat, University of Toronto, Canada. RET/PTC (TPC1) and BRAF (BCPAP, 8305C, KTC1) mutation status and thyroid cell origin was confirmed as outlined (Schweppe et al., 2008), last tested in 2012. All of the experiments were performed on cells 3-8 passage numbers (parental cell lines) and 5-15 passage numbers for transduced cell lines from the time they were received. Cell lines were proven to be mycoplasma free using the MycoAlert Mycoplasma Detection kit (Lonza, Allendale, NJ, USA). Primary cell culture and low passage number experimental cell lines were maintained in DMEM media supplemented with 10% FBS.

Isolation of primary thyroid cancer cell. Primary thyroid cancer cells were obtained using the Cancer cell Isolation Kit (Panomics, Inc., Fremont, CA, USA). Tissue was minced to small pieces under aseptic conditions, digested for 2 hr with gentle mixing at 37°C and cancer cells were purified following manufacturer's protocol. Isolated primary cancer cells were cultured in DMEM/F12 medium supplemented with 10% FBS and 6H (10 mU/mL TSH, 0.01 mg/mL insulin, 10 nM hydrocortisone, 5 μg/mL transferring, 10 ng/mL somatostatin and 10 ng/mL glycyl-L-histidyl-L-lysine).

Western blot analyses. Cells were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, 2 mM pepstatin, and 1:100 protease inhibitor cocktail set III on ice. After centrifugation at 4°C at 18,000 rpf for 15 min, the supernatant was harvested as the total cellular protein extract, aliquoted and stored at –80°C. Protein concentration was determined

using Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Aliquots (usually 50 µg) of protein extract samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane, blocked in 5% non-fat milk in TBS containing 0.05% Tween-20 for 60 min, followed by incubation with primary antibodies 4°C overnight. Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (Pierce Biotechnology, Rockford, IL, USA) and visualized with SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, Rockford, IL, USA).

Nuclear and cytoplasmic protein extractions. Nuclear–cytoplasmic fractionation was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific). Briefly, cell membranes were gently disrupted allowing for the release of the cytoplasmic contents. The intact nuclei were then recovered from the cytoplasmic extract by centrifugation. Nuclear pellets were briefly washed in PBS and extracts of nuclear proteins obtained by addition of the nuclear extraction reagent. Less than 10% contamination between nuclear and cytoplasmic fractions is expected, according to the manufacturer's protocol.

2D electrophoresis. Cell pellets containing 10^6 cells were resuspended in 140 µL of buffer containing 8 mol/L urea, 4% w/v CHAPS, 1% v/v IPG buffer (pH 3-11 NL) and 2 mg/ml DTT. Samples were centrifuged at 14,000 x *g* for 10 min at 4°C. The supernatant was loaded onto 7 cm 3-11 NL strips (GE Healthcare Bio-Sciences, Uppsala, Sweden). The strips were focused with active rehydration at 5 kV until a reading of 6.7 kV x h was reached. Strips were equilibrated prior to SDS-PAGE in 15 ml of equilibration buffer (50 mmol/L Tris·Cl pH 8.8, 6 mol/L urea, 30% v/v glycerol, 2% w/v SDS, 10 mg/mL DTT) for 15 min on a rocking platform. Strips were positioned on 4-15% SDS-PAGE gradient gels and electrophoresed at 0.2 W *per* gel for 1 hr, then at 1 W *per* gel overnight at 10°C until the bromophenol blue dye front had reached the gel bottom. To minimize variations, all gels were run simultaneously.

Short hairpin (shRNA) stable transductions. To selectively and stably knock down the expression of PDGFRα in the TPC1 and 8305C cell lines we used the HuSH-29 shRNA Vector system (HuSH-29 shRNA Retroviral Vector Systems; OriGene Technologies, Inc.). Briefly, PTC cells TPC1 and 8305C were transduced with four unique 29mer shRNA constructs in the pRS retroviral system (Puro+) followed by selection in puromycin (2.5 ug/mL). As negative control cells were also transduced with a scrambled non-effective shRNA cassette. Resistant cells were assessed by western blot to select the sequences that produced the highest levels of protein expression knock-down. The unique sequences tried were as follows:

1. GATGCCTGGCTAAGAATCTCCTTGGAGCT
2. AGTTCCACCTTCATCAAGAGAGAGGACGA
3. TCTACTTTCTACAATAAGATCAAGAGTGG
4. GAAGCAGGCTGATACTACACAGTATGTCC

sequences 1 and 2 provided the highest levels of protein expression knock-down the TPC1 and 8305C cell lines respectively and were used throughout this study. The transduced 8305C cells were further sorted by flow cytometry to select a strongly negative PDGFRα population. To stably knock down the PDGFRβ receptor, cells were transduced with the pGFP-BR-S shRNA retrovirus system (BSD+) followed by selection in blasticidin (500ug/mL). The following sequences were assessed:

1. ACCTTCTCCAGCGTGCTCACACTGACCAA
2. GACGGAGAGTGTGAATGACCATCAGGATG
3. GAGAGCATCTTCAACAGCCTCTACACCAC
4. TGCCTCCGACGAGATCTATGAGATCATGC

Resistant cells were again assessed by western blot to select the sequences that produced the highest levels of protein expression knock-down. The sequence TGCCTCCGACGAGATCTATGAGATCATGC rendered the highest level of inhibition of PDGFRβ expression in the TPC1 cell line.

Gene transfer. To express human PDGFRα complementary DNA, we used a doxycycline-inducible retrovirus system (Lenti-X Lentiviral Expression Systems; Clontech Laboratories, Inc., Mountain View, CA, USA). Briefly, PTC cells were first transduced with the LVX-Tet-On advanced lentivirus (Neo+) followed by selection in G418 (1.0 mg/mL). Resistant cells were then transduced with the LVX-Tight-Puro (Puro+) vector or sequence-verified derivatives expressing wild-type human PDGFRα complementary DNA, followed by selection in puromycin (2.5 µg/mL). Complementary DNA expression was induced by addition of doxycycline (2 µg/mL). To express PDGFRα in the rat cell line FRTL-5, the human cDNA sequence was inserted into pLenti-C-mTagGFP (SgfI/MluI) following transduction with lentiviral particles cells were sorted by flow cytometry and the GFP positive population

was cultured. Complementary DNA expression was further confirmed by western blot detection of the PDGFRα protein.

Wound healing, clonogenic, transwell invasion and proliferation assays. Cytoselect^{TM} 24-well cell invasion basement membrane assay kit (Cell Biolabs, San Diego, CA, USA) was used to measure the invasive properties of the cells. Briefly, the stable TPC1, 8305C and BCPAP cell lines were seeded at a density of $3x10^5$ cells / well and cultured for 48 hours. Invasive cells passed through the basement membrane layer, dissociated using detachment buffer and then quantified by means of CyQuant GR fluorescent dye. Adherent colony formation assays were performed as described. Fifty or 100 cells per well were plated in six-well plates, fed 5% FBS supplemented growth medium and allowed to form colonies for 20 days. Colonies were stained with 0.5% crystal violet solution in 25% methanol and counted. For the wound healing assay, cells were plated in 6 well plates at 80-90% confluence. A wound was created by manually scratching the cell monolayer with a p1000 or p200 pipet tip. Cellular debris was removed by washing the monolayer with PBS and the cells were fed with complete growth medium or serum-free medium. Images and measurements were acquired at times 0, 20 and 44 hours after wound creation. To document the effect of PDGFRα or β on proliferation, cultures were incubated in regular or serum-free-medium and enumerated daily for 5 days with an electronic cell counter (Coulter Model Zf). The MTS assay (Promega, Madison, WI, USA) was also performed in 8-16 replicates after 48 and 72 hours of growth.

Confocal microscopy. Cells were seeded on coverslips, fixed in 4% paraformaldehyde/PBS for 1 h, permeabilized with 0.5% Triton X-100/PBS for 10 min, washed in glycine/PBS for 10 min and blocked with 5% goat serum. Samples were then incubated overnight at 4°C with primary antibodies as indicated, washed and incubated for 2 hours with secondary antibodies. Cell nuclei was counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and samples were mounted with Prolong Gold Anti-fade reagent (Life Technologies). Images were taken with a Zeiss LSM 710 Axio Observer inverted 34-channel confocal microscope and analyzed with Zeiss Zen software.

Mouse xenograft models. All experiments were conducted in accordance with the guidelines of the University of Alberta Animal Care and Use Committee (ACUC) and the Canadian Council of Animal Care. SCID beige, 8-10 weeks old male mice were purchased from Charles River Laboratories, and allowed to acclimate for 5 days. Cells were maintained in DMEM media supplemented with 10% fetal bovine serum prior to inoculation. BCPAP cells (1×10^6) expressing PDGFRα protein or empty vector (mock) were inoculated subcutaneously (1:1 v/v matrigel–PBS) on the left and right flanks (respectively) of beige SCID mice that received a 0.5 mg slow release doxycycline pellet, subcutaneously 48 hours prior to cell inoculation. The stable TPC1 and 8305C cells (1×10^6) were inoculated subcutaneously as described above. Where noted, animals were treated daily with vehicle (peanut oil) or crenolanib (8 mg/kg), administered daily at 10 uL (0.8mg/mL) per gram body weight starting 24h post inoculation until termination (day 19). Tumor growth was followed and documented and animals were sacrificed once the tumors reached a $1cm^3$ size.

Sodium iodide uptake. Ex vivo measurements of sodium iodide transport in normal thyroid tissue as well as papillary thyroid carcinomas were performed both as direct measurement of radioactive iodide uptake and using a colorimetric iodide assay (Waltz et al., 2010; Weiss et al., 1984). Briefly, 50,000 cells/well were seeded on poly-L-Lys or collagen-coated 96-well plates and allowed to attach overnight. The rat cell line FRTL-5 was used as positive control for all experiments. Cells were washed twice in iodide uptake buffer (10mM HEPES/HBSS). After the final wash, 80 µL of uptake buffer was added to all wells and further supplemented with 10 µL of 100 µM NaI solution (uptake wells), 10 µL of 100 µM NaI/450 µM $NaClO_4$ solution (uptake inhibition wells) or 10 µL of uptake buffer (background control wells). Plates were incubated for 1 hr at 37°C, 5% CO_2 atmosphere then the solution was completely removed from wells and plates allowed to dry by blotting on paper towel. Water (100 µL) was added to all wells followed by 100 µL of 10.5 mM Ammonium Cerium(IV) Sulfate and 100 µL of 24 mM Sodium Arsenite(III) solution and plates were incubated in the dark for 30-90 min at RT, absorbance readings (420 nm) were taken at 30 min intervals.

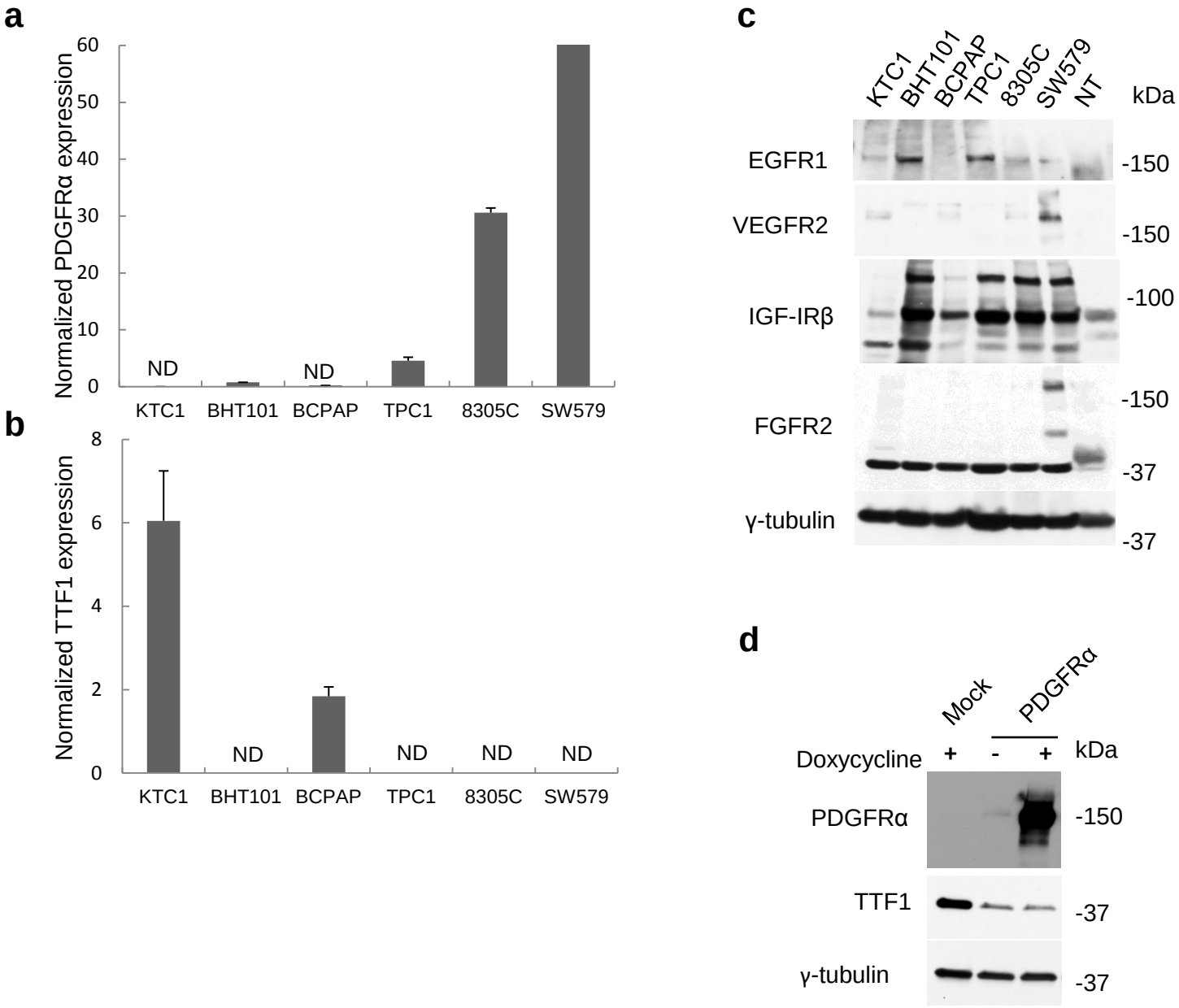
Immunohistochemistry. Briefly, formalin-fixed, paraffin-embedded tissue sections of 4 µm thickness were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed using citrate buffer (pH 6.0) and pressure-cooked in a microwave for 20 min. The endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 10 min. Tissue sections were incubated with primary antibodies overnight at 4°C, washed in PBS buffer (×2), and then incubated with biotinylated universal secondary antibody and subsequently with streptavidin–HRP complex according to the manufacturer's instructions (LSAB+ system, Dako, Burlington, Canada). Rabbit IgG

isotype control (Cell Signaling Technology) was used as the negative control for IHC staining. Tissue sections were incubated with 3,3′-diaminobenzidine/H_2O_2 (Dako) and counter-stained with haematoxylin. Evaluation of immunostaining was performed without knowledge of the clinical outcome and all specimens had representative sections confirming that more than 90% of the specimen consisted of papillary thyroid carcinoma. Sample cores on the tissue array that were fragmented or incomplete were not scored. As described in multiple reports, the cytoplasmic staining of PDGFRα and PDGFRβ was assessed for each case, in triplicate, as 3+, (strong, diffuse), 2+ (strong, focal), 1+ (weak staining), or 0 (minimal staining) (Zhang et al., 2012; Gonzalez-Campora et al., 2011; Barreca et al., 2011).

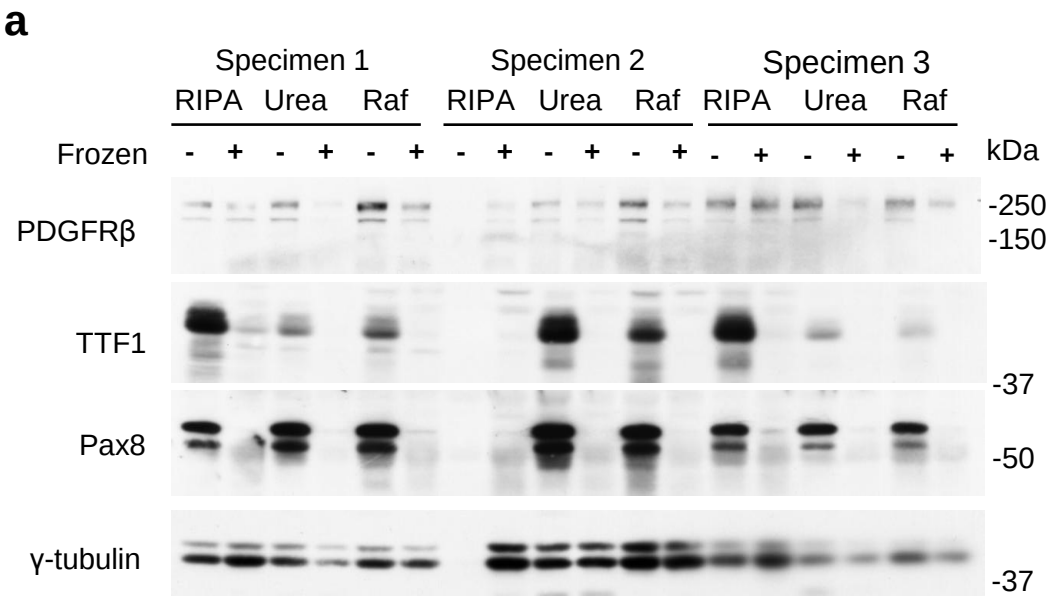
Cell cycle analysis. Cells were synchronized by a double thymidine block. Briefly, cells were exposed to two sequential incubations in 2mM thymidine each exposure separated by a 12 hr interval. After the second block, cells were released, stained with propidium iodide at fixed time intervals of 0, 4, 6, 12, 16, 18, 20 and 24 hr and subsequently analyzed by flow cytometry.

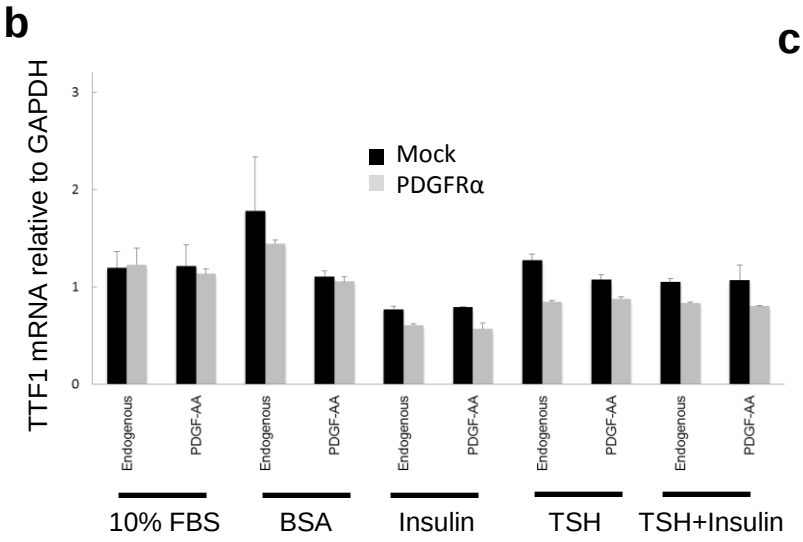
Statistical analysis. Data were expressed as the mean ± standard error of mean from a minimum of three independent experiments. Statistical analyses were performed using the two-tailed Student's *t* test for unpaired samples, with equal variance. The correlations between protein expression and metastatic status were assessed using Fisher's exact test for tables and Spearman rank correlation for continuous variables. Statistical tests are two-tailed with a P value <0.05 considered to be statistically significant. Descriptive statistics were used to present the study variables. Mean and standard deviation were reported for the continuous data variables, frequency and percentages were reported for categorical variables. Recurrence free survival (RFS) was calculated from the date of treatment to date of recurrence and the patients who did not recur were considered censored for the analysis. Kaplan-Meier methods were used to time to event data, and the median survival and the corresponding 95% confidence interval were reported. When the median survival was not reached, then the survival probabilities were reported. Log rank tests were used to compare the two survival curves. All statistical analysis was conducted in SPSS version 15. A p-value <0.05 was used for statistical significance.

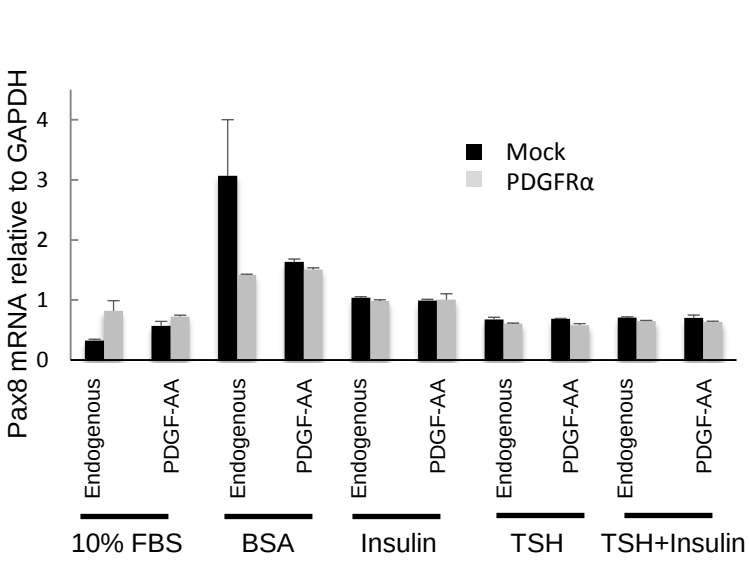
SUPPLEMENTARY FIGURES

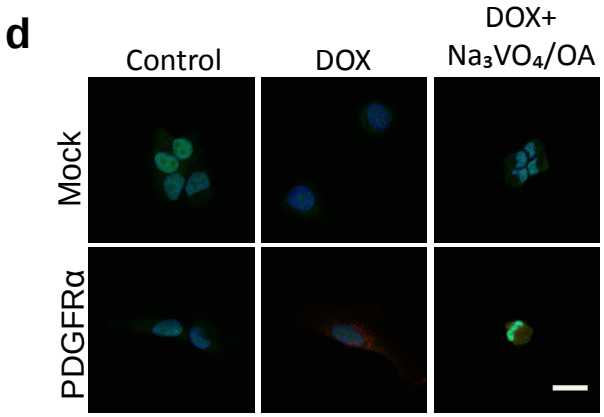


Supplementary Figure 1.
Comparative, quantitative PCR analysis of mRNA levels in papillary thyroid carcinoma cell lines confirming inverse relationship between PDGFRα (a) and TTF1 (b). Values relative to GAPDH. ND: Not detected. Data are presented as mean ± SEM. (c) Western blot analysis of representative sections of growth factor receptors EGFR, FGFR, VEGFR, and IFGR protein expression in PTC cell lines. (d) Western blot demonstrating that forced expression of PDGFRα in histologically normal thyroid tissue decreased TTF1 expression.

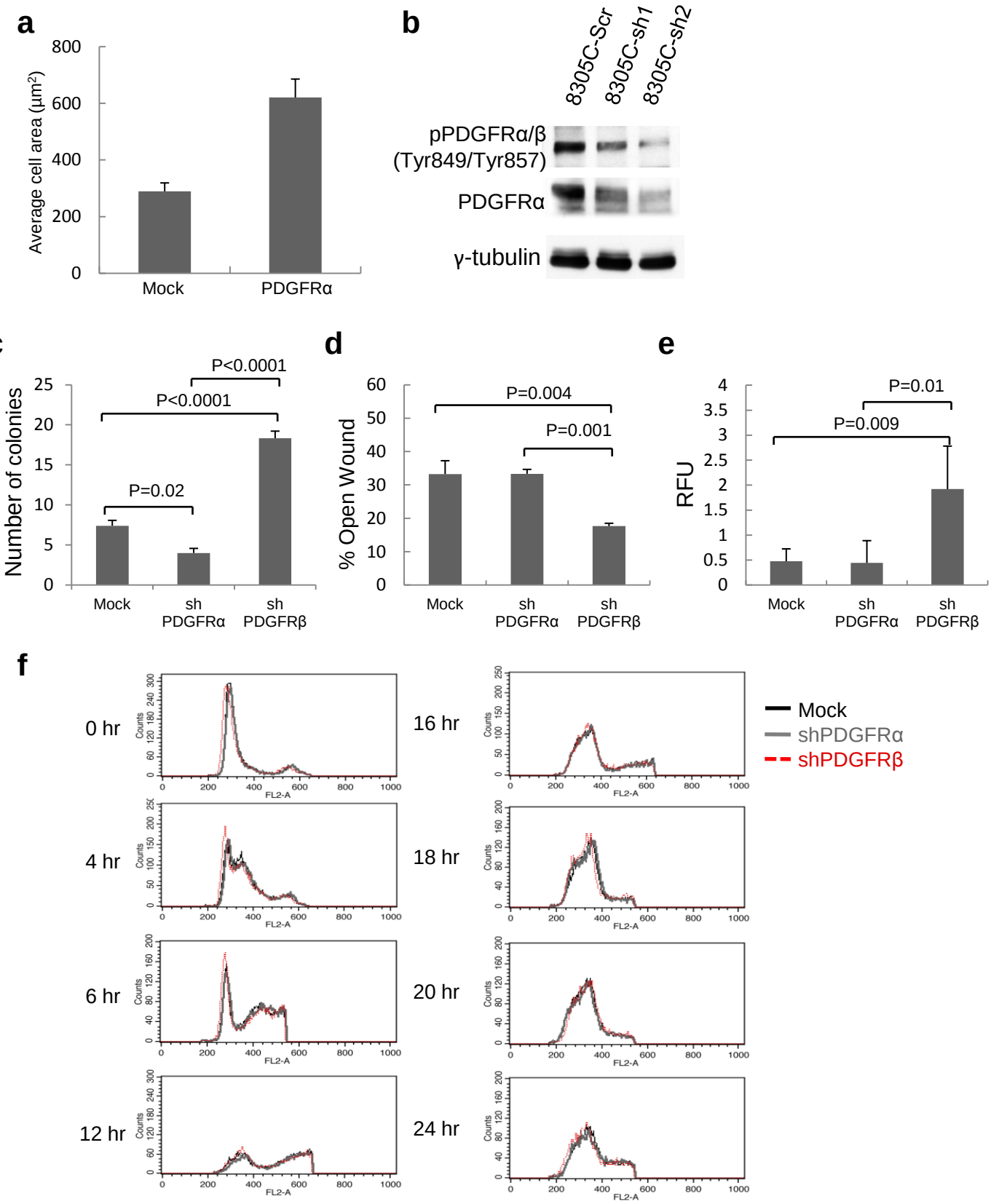






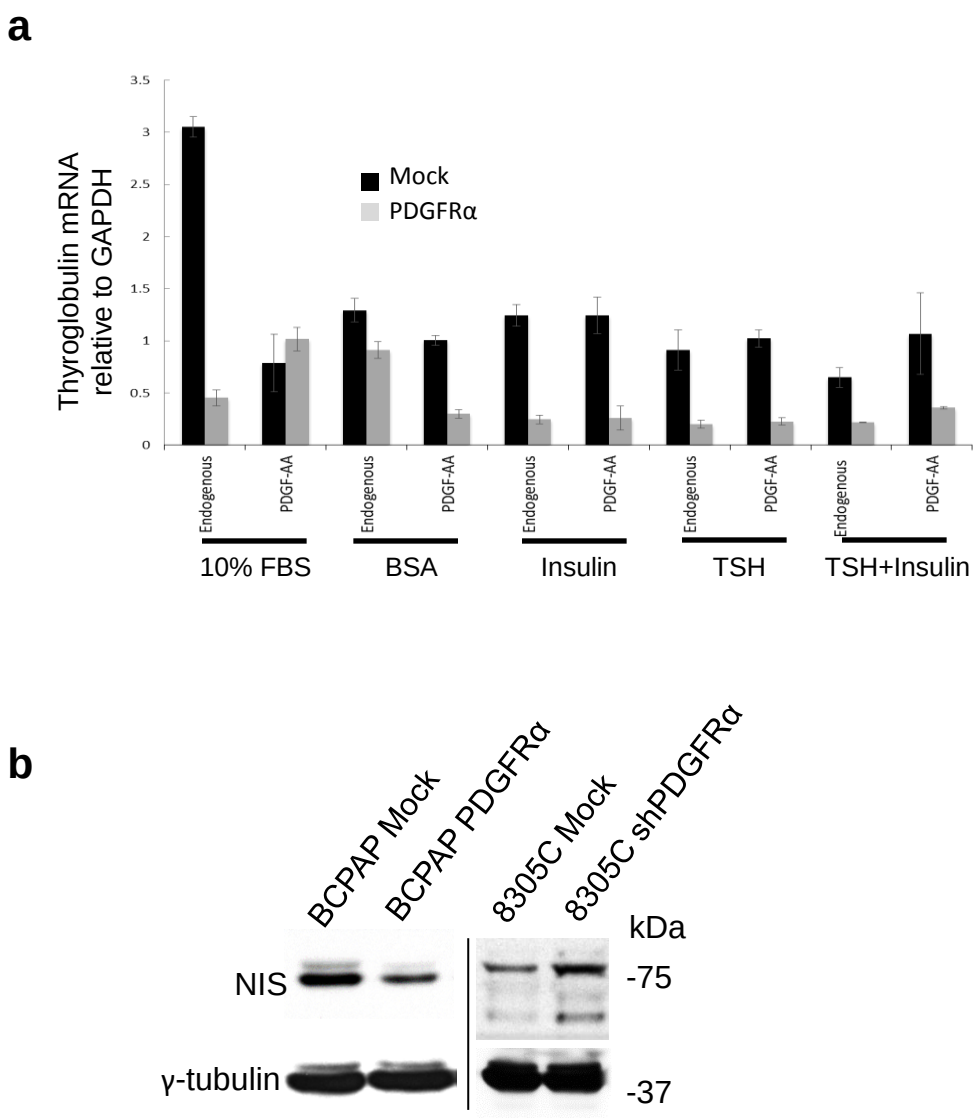


Supplementary Figure 2.
(a) Loss of nuclear factors after tissue freezing shown by Western blot comparing different tissue isolation protocols with and without snap freezing of human tissue prior to protein isolation. Comparative qRT-PCR levels of TTF1 (b) and Pax8 (c) in BCPAP cells with and without PDGFRα grown under the varying conditions including TSH, insulin, and serum supplementation. Values relative to GAPDH. Data are presented as mean ± SEM. (d) Nuclear targeting of TTF1 in both mock and PDGFRα+ BCPAP cells after treatment with phosphatase inhibitors Sodium orthovanadate (Na_3VO_4) and Okadaic acid (OA) for 2 hours. Scale bar 25 µm.

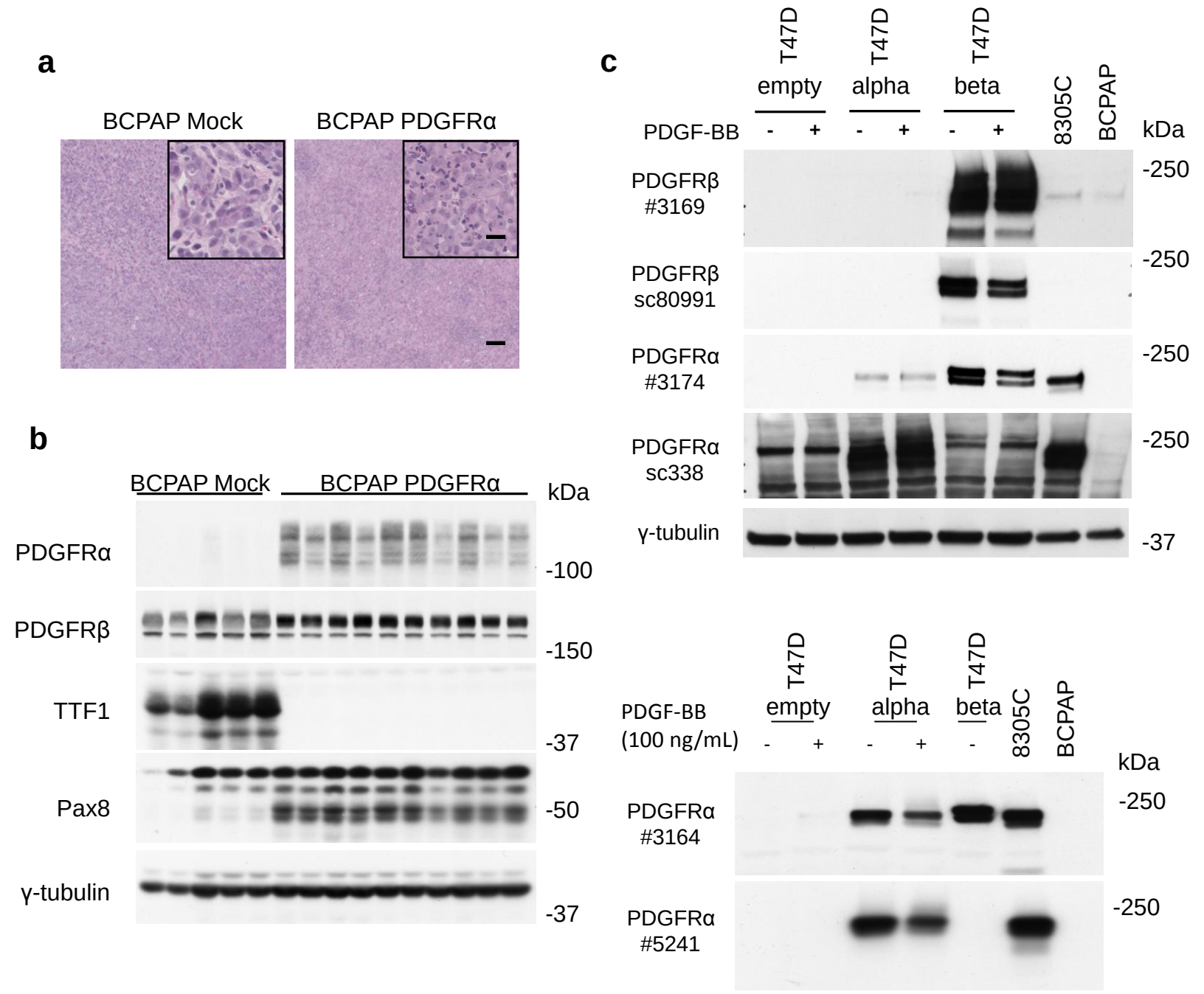


Supplementary Figure 3.

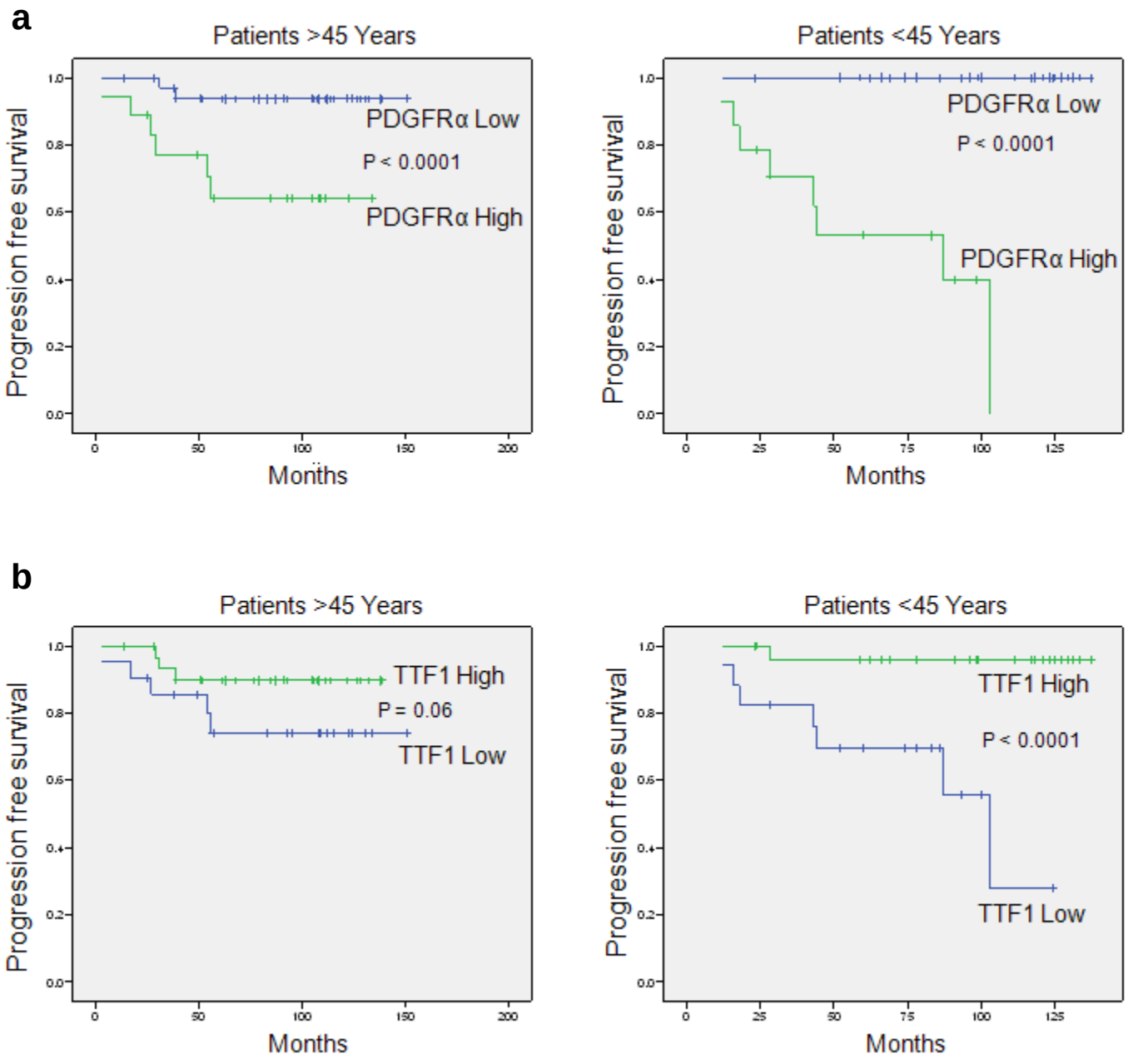
(a) Cell area quantification of BCPAP (P=0.0003) with and without PDGFRα. (b) Western blot demonstrating the selective knockdown of PDGFRα in thyroid cancer cell line 8305C using two different shRNA constructs. Negative control: Scr, non effective shRNA cassettte. TPC1 cell lines with selective knockdown of PDGFRα or PDGFRβ were assessed for colony formation (c), wound closure (d) and invasive potential (e). Data are presented as mean ± SEM. (f) Cell cycle analysis with respect to selective knockdown of PDGFRα or PDGFRβ in the TPC1 cell line.



Supplementary Figure 4.
(a) Comparative qRT-PCR levels of thyroglobulin in BCPAP cells with and without PDGFRα under varying growth conditions including TSH, insulin, and serum supplementation. Values relative to GAPDH. Data are presented as mean ± SEM. (b) Western blot of NIS protein levels in BCPAP and 8305C cells with and without PDGFRα.



Supplementary Figure 5.
(a) Representative H&E stains of the tumors derived from BCPAP cell lines with and without PDGFRα. Scale bars are 400 μm and 50 μm (inset). (b) Western blot analysis of representative sections of tumors demonstrates an inverse relationship of TTF1 and PDGFRα expression. (c)Western blots of different commercially available PDGFRα antibodies in our T47D synthetic system with selective expression of either the alpha or beta subunits of PDGFR. There is significant cross-reactivity between the beta and alpha subunits of PDGFR for most antibodies with the exception of the Cell Signaling clone 5241 for PDGFRα and Santa Cruz clone 80991 for PDGFRβ. These antibodies were subsequently optimized for immunohistochemical and western blot use for all experiments on human tissue.



Supplementary Figure 6.
Kaplan-Meier plot of disease recurrence as a function of time and age ((>45 years , left panels and < 45 years, right panels) based on PDGFRα (a) and TTF1 (b) immunohistochemical staining.

SUPPLEMENTARY TABLES

Supplementary Table 1. Scoring summary for immunohistochemistry

Marker	Tissue	Site and Intensity (%)			
TTF1		**Nuclear**			
		0	**1**	**2**	**3**
	Normal thyroid (n=36)	3	8	11	78
	Benign thyroid (n=57)	4	12	21	63
	Node-negative primary (n=113)	15	13	31	40
	Node-positive primary (n=68)	25	39	19	18
	Lymph node metastases (n=13)	62	15	8	15
		Cytoplasmic			
		0	**1**	**2**	**3**
	Normal thyroid (n=36)	94	6	0	0
	Benign thyroid (n=57)	79	14	7	0
	Node-negative primary (n=113)	62	28	10	0
	Node-positive primary (n=68)	37	35	24	4
	Lymph node metastases (n=13)	38	44	19	0
Pax8		**Nuclear**			
		0	**1**	**2**	**3**
	Normal thyroid (n=36)	3	8	8	81
	Benign thyroid (n=57)	2	7	24	66
	Node-negative primary (n=113)	9	11	19	61
	Node-positive primary (n=68)	20	13	12	55
	Lymph node metastases (n=13)	15	23	31	31
		Cytoplasmic			
		0	**1**	**2**	**3**
	Normal thyroid (n=36)	77	19	3	0
	Benign thyroid (n=57)	63	23	12	2
	Node-negative primary (n=113)	61	16	20	2
	Node-positive primary (n=68)	53	34	9	4
	Lymph node metastases (n=13)	54	31	15	0
		Cytoplasmic			
PDGFRα		**0**	**1**	**2**	**3**
	Normal thyroid (n=36)	84	8	8	0
	Benign thyroid (n=57)	78	9	8	4
	Node-negative primary (n=113)	77	11	9	3
	Node-positive primary (n=68)	17	17	40	26
	Lymph node metastases (n=13)	15	23	38	23

Supplementary Table 2. Tissue array distribution

Tissue	Age (mean)	Female (male)	Tumor size (cm)	Nodes assessed (mean)
Normal (n=36)	47.1 ±14.8	29 (7)	-	-
Benign neoplasms (n=57)	51.7 ± 14.6	37 (20)	3.5 ± 1.8	0.6 ± 1.5
Node positive (n=68)	45.9 ± 15.9	53 (15)	2.6 ± 1.5	22.2 ± 23.2
Node negative (n=113)	48.4 ± 15.3	93 (20)	2.3 ± 1.8	3.9 ± 9.3
Lymph node metastases (n=13)	48.1 ± 13.1	10 (3)	2.5 ± 2.7	15.3 ± 13.6