Targeting a Trigger: Platelet Derived Growth Factor Receptor Alpha in the Nodal Metastasis of Papillary Thyroid Cancer

by

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ABSTRACT

Recurrent, metastatic forms of papillary thyroid cancer (PTC) continue to challenge the status quo therapy regime which combines surgical resection with radioactive iodine ablation of remnant thyroid and metastatic deposits. Repetitive use of these methods following the initial failure to control the disease impacts negatively on the quality of life and survival. Investigational use of targeted therapies like tyrosine kinase inhibitors (TKIs) have become a major part of the quaternary care given to patients with the recurrent, metastatic disease. Unfortunately, the varied outcomes and adverse side effects which accompany their empirical use underscores the need for a much better understanding of the cellular processes driving metastatic disease in PTC.

We previously identified platelet derived growth factor receptor alpha (PDGFRα) as an important biological marker for the metastatic spread of PTC. By extension, in the current work, the prognostic implication of activated PDGFRα in papillary thyroid tumor samples was evaluated. We hypothesized that PDGFRα induces aggressive phenotypes such as lymph node metastases, dedifferentiation and compromised iodide transporting abilities, in papillary thyroid tumors. The signaling consequences of PDGFRα, and the effects of its blockade by tyrosine kinase inhibitors were also investigated.

We demonstrate that PDGFRα creates an invasive and migratory follicular cell phenotype lacking the ability to transport sodium iodide in vitro and in vivo. The invasive and migratory behaviour are consequences of cellular epithelial to

mesenchymal transition, and the formation of ECM degrading invadopodial protrusions. The unique ability of PDGFRα to mediate thyroid follicular cell dedifferentiation, with the ensuing suppression of iodide uptake in PTC cells, results from the downregulation of TTF1 expression. In clinical samples, PDGFRα is predictive of nodal spread, radioactive iodine resistance, as well as disease recurrence. We also observed that the expression of PDGF-AA, a PDGFRα-specific ligand, is associated with the metastatic PTC disease. Interestingly, our findings challenge the current canonical description of PTC as a MAPK/ERK driven malignancy, and clearly demonstrate a vital role for the STAT3 and AKT pathways in driving this aggressive disease phenotype.

PDGFRα represents the first opportunity for targeted therapy in thyroid cancer that directly influences tumor differentiation, migration and ECM degradation. We propose that blockade of PDGFRα may be a useful adjunct therapy given its ability to dramatically reduce the severity of the aggressive phenotypes, where previous attempts with drugs that disrupt other tyrosine kinases have failed.

PREFACE

The work in this thesis was undertaken under the supervision of Dr. Todd P. W. McMullen. Human specimens were obtained with approval of the University of Alberta Health Research Ethics Board ("Mechanisms of Lymph Node Metastases", ID Pro00018758, 01/15/2011).

Chapter 3 is part of a manuscript prepared as: **Esther Ekpe-Adewuyi,** Sunita Ghosh, Todd P. W. McMullen. The expression of Platelet-Derived Growth Factor-AA and -α Receptor is prognostic of nodal infiltration in Papillary Thyroid Cancers. I performed all the experiments. Sunita Ghosh assisted with the statistical analysis.

Chapter 4 is part of a manuscript prepared as: **Esther Ekpe-Adewuyi,** Ana Lopez-Campistrous, Xiaoyun Tang, David N. Brindley, Todd P. W. McMullen. Platelet Derived Growth Factor Receptor Alpha Drives the Epithelial to Mesenchymal Transition Creating a Migratory Phenotype in Papillary Thyroid Cancer. I performed and analyzed all of the experiments. Ana Lopez-Campistrous established the stably transfected PTC cells. Xiaoyun Tang assisted with migration assays.

Chapter 5 is part of a manuscript prepared as: Ana Lopez-Campistrous, **Esther Ekpe-Adewuyi**, Matthew GK Benesch, Raymond Lai, Aducio Theisen, Jay Dewald, Peng Wang, David C Williams, Larissa J Vos, David N Brindley and Todd P. W. McMullen. Platelet Derived Growth Factor Receptor Alpha is a Master Switch for

Dedifferentiation Driving Lymphatic Metastases in Papillary Thyroid Cancer. I performed all immunohistochemical staining and imaging of clinical samples. Todd P. W. McMullen performed the Kaplan-Meier analyses. 3D matrigel assays, flow cytometry as well as western blots on clinical samples were done by me. Ana Lopez-Campistrous and I propagated the primary cell cultures and performed the colorimetric iodide uptake assays.

DEDICATION

To God be the Glory, His Grace and Mercy brought me through

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To Dr Todd McMullen: Thank you for taking me on, I am so grateful to have been a part of the McMullen Lab. I feel fortunate to have had your unwavering support and guidance through to the end. I thank you for the time and energy you committed to making me a better scientist and researcher. I have learned so much from your ambition and enthusiasm. Thanks for being a true mentor.

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LIST OF ABBREVIATIONS

PTC: papillary thyroid cancer

PDGF: platelet derived growth factor

PDGFR: platelet derived growth factor receptor

TSH: thyroid stimulating hormone/thyrotropin

TTF1: thyroid transcription factor 1

NIS: sodium/iodide symporter

PAX8: paired box gene 8

ERK: extracellular-signal-regulated kinase

PI3K: phosphatidylinositol 3-kinase

MAPK: mitogen-activated protein kinase

STAT3: signal transducer and activator of transcription 3

MMP: matrix metalloproteinase

TKI: tyrosine kinase inhibitor

EMT: epithelial to mesenchymal transition

RAI: radioiodine/ 131I

ECM: extracellular matrix

IHC: immunohistochemistry

qRT-PCR: quantitative real-time polymerase chain reaction

GAPDH : glyceraldehyde 3-phosphate dehydrogenase

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

shRNA: short hairpin ribonucleic acid

TBS: tris-buffered saline

SEM: standard error of the mean

3D: 3 dimensional

CHAPTER ONE: INTRODUCTION

1.1 Thyroid

The thyroid gland, which is the largest endocrine organ in humans, regulates systemic metabolism through thyroid hormones. This butterfly-shaped organ made up of lobules of spherical follicles has two distinct hormone-producing cell types designated follicular and para-follicular C cells. The follicular cells which are the most abundant, are epithelial in nature and vary in shape from cuboidal to columnar. They are responsible for iodide uptake and syntheses of the thyroid hormones, L-triiodothyronine (T3) and L- thyroxine (T4). Structurally, the follicular cells form distinct colloid-containing follicles full of thyroglobulin which serve as the supply pool during thyroid hormone synthesis (Fig. 1.1A). Hematoxylin and eosin staining of a normal thyroid section with follicles of various sizes surrounded by follicular cells is shown in Fig. 1.1B. Overall, the synthesis, storage and secretion of T3 and T4 are under the control of the hypothalamic-pituitary axis with negative feedback from the hormones. Thyrotropin releasing hormone which is secreted from the hypothalamus stimulates the release of thyroid stimulating hormone (TSH) from the anterior pituitary gland, and TSH stimulates the follicular cells to synthesize and secrete T3 and T4. In much smaller numbers are the neuroendocrine-originating para-follicular cells, which are dedicated to the production of calcitonin, the calcium-regulating hormone (1).

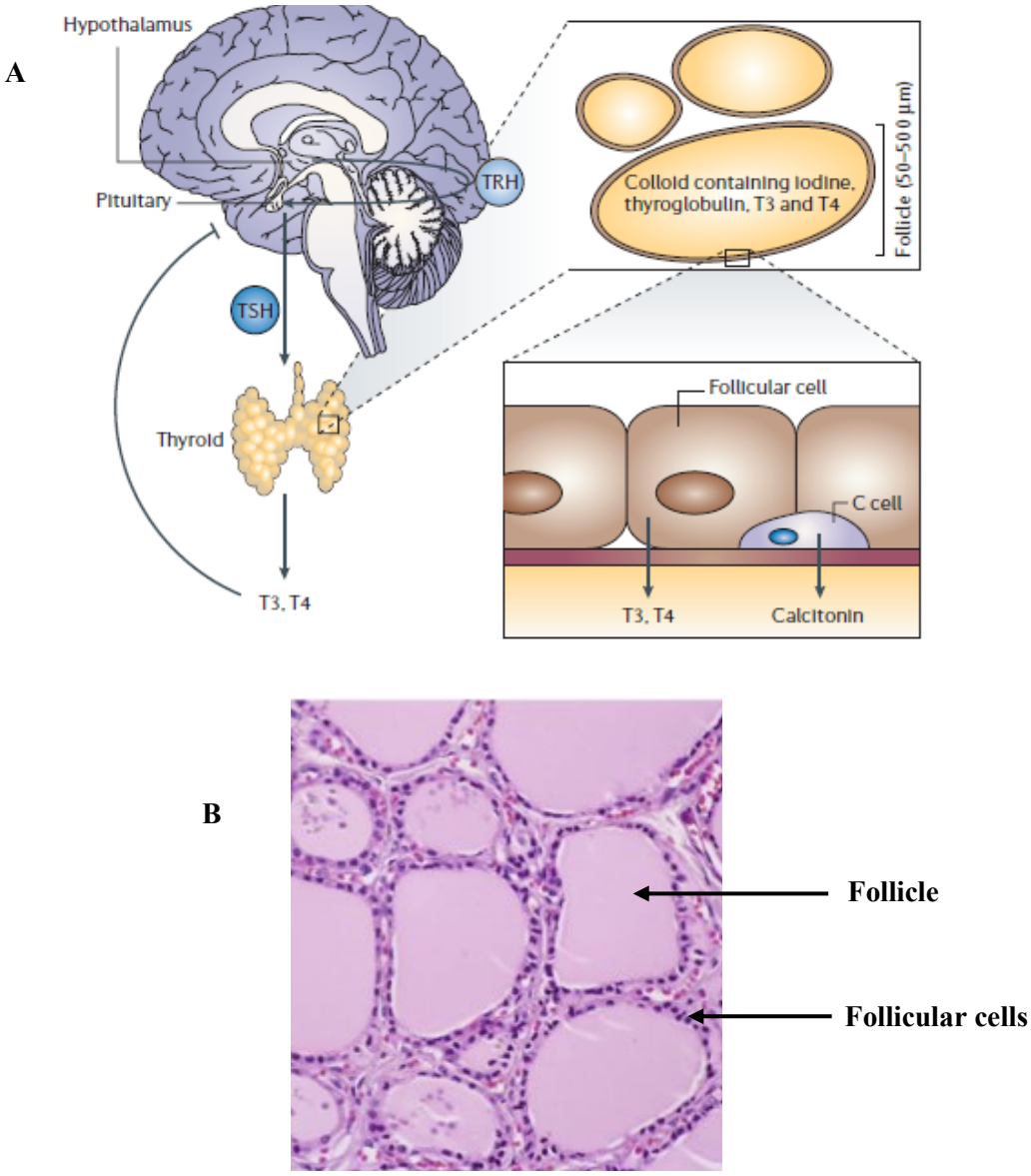

Figure 1.1: The structural and functional schematic of the thyroid gland. (A) The thyroid is gland is made up of colloid-containing follicles of varied sizes surrounded by follicular cells. The follicular cells synthesize, store and secrete thyroid hormones T3 and T4 under the control of the hypothalamic-pituitary axis with negative feedback by the thyroid hormones. The C cells which produce calcitonin are found in the intra-follicular spaces. Images were taken from Ref. 1. **(B)** Hematoxylin and Eosin staining of a normal thyroid section showing follicles of various sizes surrounded by follicular cells.

1.1.1 Differentiation of Thyroid Follicular Cells

The differentiation of thyroid follicular cells (TFCs) occurs during morphogenesis, a process that involves the migration of endodermal cells of the primitive pharynx to the position occupied by the adult thyroid at the base of the neck. Due to the ease of cultivating them as primary cultures or stable cell lines, TFCs have even become a model for in depth study of the molecular mechanisms involved in epithelial cell differentiation and maintenance (2). Functional differentiation of TFC is initiated with the expression of thyroid-specific genes whose protein products are essential for thyroid hormone synthesis and release into the blood stream (2). These thyroid-specific genes are either uniquely expressed in the TFCs, such as those encoding thyroglobulin (TG), thyroperoxidase (TPO), or expressed in a very restricted number of cell types, as in the case of TSH receptor (TSHR), and the sodium/iodide symporter (NIS) proteins required for the synthesis of the thyroid hormones (2). Sustained expression of the thyroid-specific genes and maintenance of the differentiation status is dependent on the coordinated action of a set of transcriptional regulators which includes the thyroid transcription factor 1 (TTF1), the paired-box transcription factor (PAX8), and the thyroid transcription factor 2 (TTF2) (3). The properties of the transcription factors are shown in Table 1.1.

During mouse development, the follicular cell precursors begin to express TTF1, TTF2 and PAX8 at embryonic day 9 (E9), while the expressions of TG, TPO, NIS and TSHR only become detectable after E14.5 (3). The proposed mechanism of developmental regulation of thyroid differentiation in the mouse as inferred from

currently available data is shown in Fig. 1.2. The thyroid phenotypes in TTF1, TTF2 and PAX8 knockout mice are shown in Table 1.2.

Table 1.1: Properties of the thyroid specific transcription factors
(Adapted from Ref. 4)

Properties of the Thyroid-Specific Transcription Factors					
Transcription factors	**Chromosomal localization**		**DNA-binding domain**	**Site of expression**	
	Mouse	**Human**		**Embryo**	**Adult**
TTF-1	12	14q13	Homeodomain	Thyroid	Thyroid
				Lung	Lung
				Forebrain	
				Pituitary	
TTF-2	4	9q22	Forkhead	Thyroid	Thyroid
				Anterior	
				Pituitary	
PAX-8	2	2ql2	Paired	Thyroid	Thyroid
				Kidney	(Kidney)
				Mid-hindbrain	
				Boundary	

Table 1.2: Thyroid Phenotypes in TTF1, TTF2 and PAX8 knockout mice
(Adapted from Ref. 2)

Transcription factors	**Role in thyroid development**	**Thyroid phenotype in null mice**
TTF-1	Proliferation and survival of thyroid follicular cells and C cells	Athyreosis – absence of whole gland
TTF-2	Migration of thyroid primordium	Ectopy of thyroid gland and reduction in size
PAX-8	Proliferation and survival of thyroid follicular cells	Absence of thyroid follicular cells

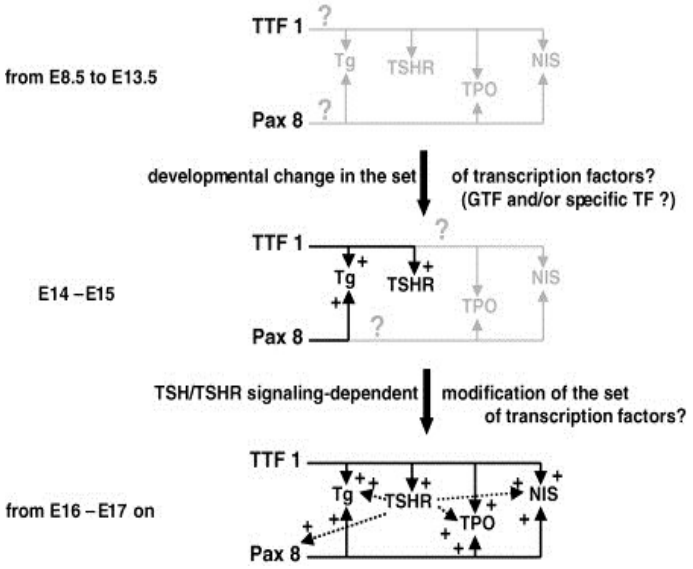

Figure 1.2: The proposed scheme of developmental regulation of thyroid-specific gene expression in the mouse as inferred from existing literature. Arrows correspond to either the known interactions between an activating transcription factor and gene promoter elements (TTF1, PAX8: **full lines**), or an observed but uncharacterized indirect effect (TSHR: **dotted lines**). Actual activations are indicated by dark arrows marked with a plus character. Grey arrows correspond to the binding of transcription factors to gene promoter elements without concomitant transcriptional effect. In the earlier stages of thyroid development form E8.5 - E13.5 **(top)**, the existing transcription factors TTF1 and PAX8 are both unable to activate the expression of the TSHR, NIS, TPO and TG genes. Whether they are already able to bind to the promoter elements of their target genes remains to be determined. The appearance or disappearance of still uncharacterized factor(s), either a general transcription factor (GTF) or a specific transcription factor (TF), is hypothetically required for transcriptional activation of the TSHR and TG genes, but is not sufficient to activate the NIS and TPO genes **(middle)**. The emergence of an active TSH/TSHR signaling is supposed to trigger other changes in the transcription apparatus resulting in the activation of the NIS and TPO genes, and in a modified control on PAX8 and TG genes expression **(bottom)**. This figure was taken from Ref. 3.

1.2 Classification of Thyroid Cancers

The vast majority of thyroid tumors arise from the thyroid follicular epithelial cells, while only about 3% of them originate from the parafollicular C cells. Cancers that originate from the parafollicular C cells are designated medullary thyroid carcinomas (MTCs) (5, 6). Follicular derived carcinomas are subdivided into well-differentiated, poorly differentiated and undifferentiated (anaplastic; ATC) carcinomas. For some follicular derived carcinomas, follicular thyroid adenoma (FTA) a benign tumor is sometime the precursor. Papillary (PTC) and follicular (FTC) are sub-types of the well-differentiated cancers which still display some or all of the characteristics of mature thyroid tissue (5, 6). These histological forms of thyroid tumors and their characteristics are summarised in Table 1.3. PTC is the most common type of thyroid cancer accounting for 85% of all diagnoses, followed by FTC (~10%) which is characterized by a poorer prognosis than PTC. ATC which accounts for only 2–3% of all cases is the most aggressive type of thyroid cancer, spreading quickly and it is difficult to treat (5, 6). PTC will be the focus of this thesis.

Table 1.3: Histological classifications of thyroid cancers and their features (adapted from Ref. 6)

Tumor type	Cell of origin	Prevalence (%)	Standard care and prognosis	Characteristics
FTA	Follicular thyroid cells (which produce thyroid hormone and thyroglobulin)	Benign lesion	Conservative monitoring; thyroidectomy if symptomatic	Common benign thyroid tumor; similar architecture to FTC, but typically encapsulated; lacking capsular or vascular invasion; lacking metastasis; lacking nuclear features of PTC
PTC*	Follicular thyroid cells	~85	Thyroidectomy and, in selected cases, radioiodide ablation (novel drugs for resistant disease); good overall prognosis	Well differentiated, with papillary architecture and characteristic nuclear features that include enlargement, oval shape, elongation, overlapping and clearing, inclusions and grooves; propensity for lymphatic metastasis; PTC subtypes include conventional PTC (CPTC), follicular-variant PTC (FVPTC), tall-cell PTC (TCPTC) and a few rare variants
FTC*	Follicular thyroid cells	~10	Thyroidectomy and radioiodide ablation (novel drugs for resistant cases); good overall prognosis	Well differentiated, hypercellular, microfollicular patterns, lacking nuclear features of PTC; vascular or capsular invasion; propensity for metastasis via the blood stream; Hürthle cell thyroid cancer is a unique subtype of FTC that accounts for 2–3% of thyroid cancers and is characterized by large, mitochondria-rich oncocytic cells and dense nuclei and nucleoli, as well as a high propensity for metastasis and a poor prognosis
PDTC	Follicular thyroid cells	~5	Surgery, radioiodide (in selected cases), chemotherapy, radiotherapy, novel drugs; poor prognosis	Poorly differentiated, often overlapping with PTC and FTC; intermediate aggressiveness between differentiated and undifferentiated thyroid cancers
ATC	Follicular thyroid cells	2–3	Surgery, chemotherapy, radiotherapy, novel drugs, palliative care; highly and rapidly lethal	Undifferentiated; admixture of spindle, pleomorphic giant and epithelioid cells; extremely invasive and metastatic; highly lethal; may occur *de novo* or derive from PTC, FTC or PDTC
Medullary thyroid cancer	Parafollicular C cells	2–3	Surgery, chemotherapy, radiotherapy, novel drugs	Moderate aggressiveness, high propensity for lymphatic metastasis; *RET* mutation; occurring in familial, MEN2 or sporadic forms

ATC, anaplastic thyroid cancer; FTA, follicular thyroid adenoma; FTC, follicular thyroid cancer; MEN2, multiple endocrine neoplasia type 2; PDTC, poorly differentiated thyroid cancer; PTC, papillary thyroid cancer. *PTC and FTC are collectively classified as well-differentiated thyroid cancer (WDTC).

1.3 Papillary Thyroid Cancer

The global rise in the incidence of thyroid cancer over the last 40 years has been largely attributed to a tripling in the number of PTC cases. It has risen faster than that reported for any other solid tumor over the past 4 decades (7, 8). Hence,

PTC has become the most common malignancy of the endocrine system (8). As depicted in Fig. 1.3, thyroid cancer is expected to surpass colorectal cancer to become the fourth leading malignancy by 2030, in the United States (9). An estimated 64,300 new cases of thyroid cancer were expected to be diagnosed in 2016, making it the eigth most common malignancy (10). In Canada, there was a 6.3% per year increase in males since 2001 and a 4.4% per year increase in females between 2005 and 2010. Thyroid cancer is now the forth most common malignancy in Canadian females (11). Datasets across America, Asia, Europe, Oceania and Africa also confirm increased incidence rates of thyroid cancer diagnosis in most populations around the world, with the average increase being 58.1%. The rates among males and females rose by 48.0% and 66.7% respectively (9). European countries reported increases in incidence between 5.3% (Switzerland) and 155.6% (France). Globally, the highest increases were observed in New South Wales, Australia, where there was a 177.8% increase in men and 252.2% in women between 1973 and 2002 (12).

Increased incidence may be due to several reasons, including overdiagnoses. More frequent use of sensitive diagnostic procedures, such as ultrasound, computed tomography scanning and magnetic resonance imaging, may mean that more earlier stage, asymptomatic thyroid cancers are being diagnosed. There are also arguments in favor of a true increase, due to increased exposure to risk factors such as ionizing radiation and environmental pollutants (11).

The 5-year survival rate approaches 100% for localized disease, is 98% for regional stage disease, and 55% for distant stage disease (10). Despite these

encouraging survival statistics, deaths from papillary thyroid cancer are up to 30,000/year world-wide (13). While PTC can affect patients of any age, it presents mainly in adults between the ages of 30-50. It is also the predominant form of thyroid cancer in pediatrics (14). The relative survival rate declines markedly after age 65 (8). Based on this, Sherman (15) projected that without significant improvements in treatment; further increases in the mortality rate could be expected since the population of the 65 years olds and above are expected to double in the next 20 years (16).

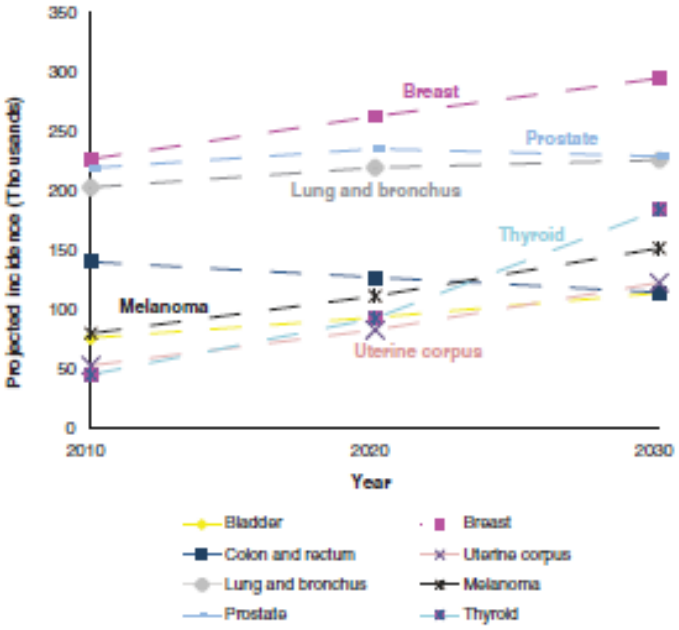

Figure 1.3: Projected cancer incidence for the United States of America. Incidence projections for the top eight cancers by 2030 due to demographic changes and the AAPC in incidence rates. All cancer sites shown have at least 110,000 cases projected by 2030 when both the demographic and AAPC factors are taken into consideration. This figure was taken from Ref. 10.

1.3.1 Risk factors

There is a genetic dimension to the risk of developing PTCs even though PTCs are usually sporadic (17). Family studies and epidemiological evaluations have provided evidence to support the existence of familial PTC forms. Susceptibility gene loci (1q21), t (3; 8) (p14.2; q24.1) and (19p13.2) have been identified in familial tumor syndromes that predispose to PTC associated with papillary renal-cell carcinoma, clear cell renal carcinoma and multinodular goitre, respectively (17).

Another well-known risk factor for thyroid cancers especially PTC is exposure to ionizing radiation (1). There are concerns that the rising incidence might be partially due to the wider use of medical radiation and increasing exposure to radiation as a result of events such as nuclear accidents, atomic bombs, and nuclear testing (18, 19, 20). It has been reported that the external beam-radiation exposure used for the treatment of benign conditions increases the risk of PTC in children (20). Studies have shown that the mechanism for radiation-induced PTC is closely linked to chromosomal rearrangement as opposed to intragenic point mutation as a mode of aberrant gene activation (19, 20).

Interestingly, the level of dietary iodide intake also predisposes to PTC development. PTC is prevalent in regions of iodide-rich diets compared to iodide-deficient areas (21). To support this epidemiological finding, laboratory studies have shown that iodide supplementation causes animal models of thyroid cancers to change from follicular to papillary morphology. Iodide is an essential requirement for thyroid hormone organification (21).

PTC is ~4 times more frequent in females than in males (22) and this gender disparity in the distribution of incidence suggests the involvement of female hormones in its pathogenesis. Although the oestrogen receptor is expressed by follicular cells, and oestrogen promotes the proliferation of these cells (23), there has been no clear-cut correlation between PTC and exogenous sex hormones (24).

1.3.2 Diagnosis and Clinical Features

PTC is first apparent as a palpable nodule within an otherwise normal thyroid and often multifocal when it occurs within a single lobe, but bilateral (occurring in both thyroid lobes) in 20-80% of cases (25). Mostly discovered incidentally or on routine examination, the incidence of suspicious nodules in the adult population of the United States ranges from 1-10%, but only about 10% of these turn out to be malignant (26). While majority of PTC may be completely asymptomatic, hoarseness, cough, dysphagia, or dyspnea in patients suggests disease advancement (26, 27).

Macroscopically, PTC tumors present as firm, granular lesions with discernible papillary structures (**Fig. 1.4A**). The lesions are often cystic, and may contain areas of fibrosis and calcification. The final diagnosis is usually based on the histopathologic evaluation of a thyroid nodule following fine-needle aspiration biopsy (FNAB). Microscopically, PTC forms branching papillae which disrupts the normal thyroid architecture, causing a major loss of the colloid structures (**Fig. 1.4B**). Psammoma bodies, which are concentrically calcified structures within the cores of the papillae, are unique to PTCs and frequently present in tumor specimens (26, 28).

The nuclei of PTC cells have a distinctive appearance which is critically important for cytologic FNAB diagnosis. Their nuclei are larger and more ovoid than those from normal thyroid follicular cells, and contain hypo-dense chromatin as well as a prominent nuclear membrane, which creates an empty appearance on hematoxylin and eosin staining (**Fig 1.4C**). The diagnosis of PTC is usually rendered in the presence of this characteristic nuclear feature (often described as Orphan Annie Eye nuclei) even in the absence of the papillae or psammoma bodies (26, 28, 29). Several histologic variants of PTC have been described based on the size or architectural pattern of the tumor (26, 28, 29). Accurate diagnosis of some of these variants is important because they have poorer prognosis compared to the classic PTC. Ancillary tests including immunohistochemcial staining and molecular detection of gene rearrangements and point mutation, allow for correct classification of borderline lesions and assist pathologists in making more accurate diagnosis (29).

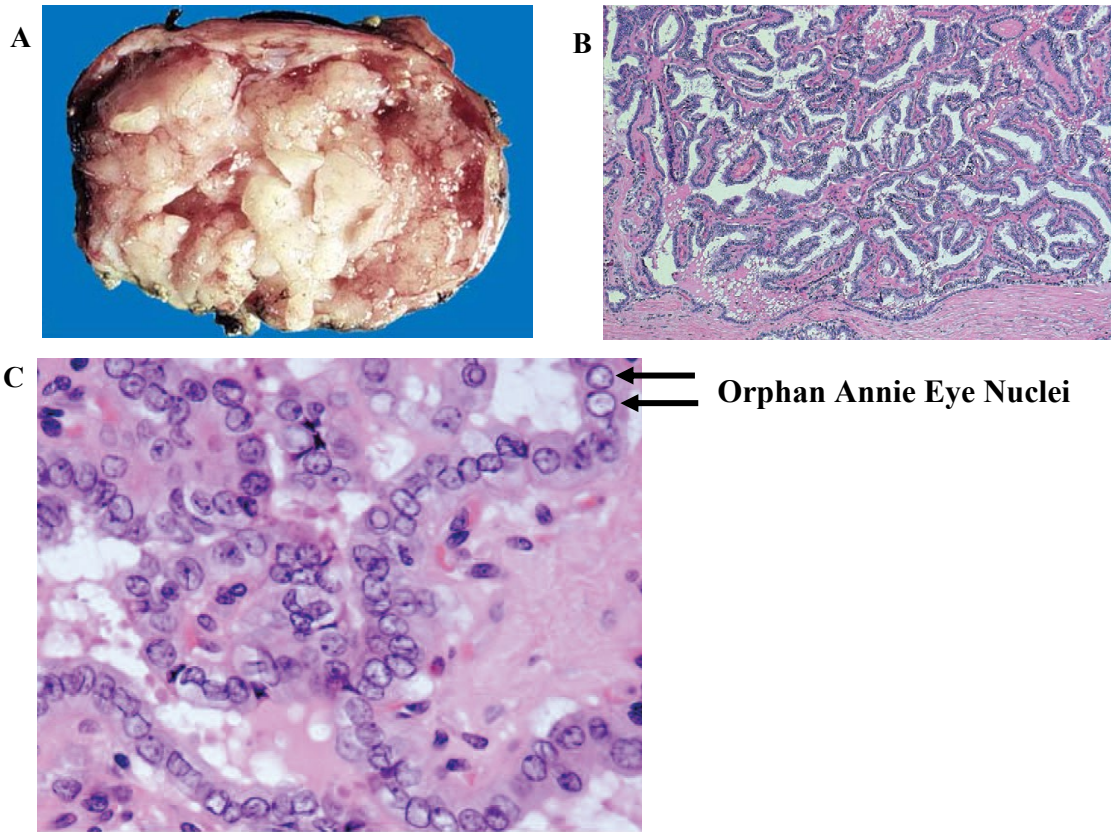

Figure 1.4: Papillary thyroid cancer. (A) The macroscopic appearance of PTC with grossly discernible papillary structures. **(B)** Hematoxylin and Eosin staining of a Classic PTC with well-formed papillae branches. **(C)** Nuclear features of PTC cells - large, empty-looking (Orphan Annie eye) nuclei often overlapping one another. Images were taken from Ref. 29.

1.3.3 Treatment

Surgical removal of the thyroid (thyroidectomy) is the first line treatment for PTC patients, even though the extent of surgical resection that provides optimal disease management remains a matter of debate (27, 30). This decision is influenced by a variety of factors, including the histopathologic diagnosis, tumor size, presence of metastases (as well as whether the metastases are lymphatic or distant), and age of the patient (25). For standardized therapeutic interventions, guidelines and recommendations from the American Thyroid Association are available (31). However, total or near-total thyroidectomy is usually favored by many surgeons (32, 33). The benefits of this radical surgical approach include reduction in recurrence rates as well as the facilitation of postoperative radioiodide treatment. In extremely low-risk patients (with unifocal tumors smaller than 1 cm, and no evidence of lymphatic or distant metastases), only the affected thyroid lobe may be removed. However, this conservative approach requires a more vigilant follow-up involving serial ultrasounds of the remaining thyroid tissue to detect any ensuing tumor (30, 32, 34). Generally, thyroidectomy promotes better prognosis in PTC patients, with 5-year survival rates and local recurrence occurring in 5-20% of patients (26). Potential complications from total or near-total thyroidectomy include transient or permanent vocal cord paralysis in ~ 1-2% of patients (25). Temporary (7-10% of patients) or permanent hypocalcemia (1-2% of patients) can also occur if the parathyroid glands, which have extremely close proximity to the thyroid, are mistakenly injured during surgery (25).

Postoperative management of PTC is intended for the elimination of residual malignant thyroid tissue or undetected metastases, and the restoration of near normal thyroid hormone activity. The former is achieved through radioiodide ^{131}I (RAI) therapy where high doses of radiation are delivered to the thyroid tissue without significant damage to other organs of the body. Radioiodide therapy also destroys any residual normal thyroid tissue (25). Secondly, total thyroidectomy carries with it the need for the patient to receive lifelong thyroid hormone replacement therapy. This is usually achieved by the administration of levothyroxine, a synthetic form of T4, which promotes normal basal metabolic activity. On the other hand, lobectomy does not usually require subsequent levothyroxine therapy (25).

1.4 Oncogenesis in PTC

Thyroid cancer is thought to be the consequence of genetic and epigenetic alterations, which affect classical signaling pathways involved in cell proliferation and survival. Perturbations in gene expression due to chromosomal rearrangements and point mutations drive thyroid oncogenesis (6). Central to PTC pathogenesis are two canonical signaling pathways, namely the mitogen-activated protein kinase (MAPK)/Erk and the phosphatidylinositol-3 kinase (PI3K)/Akt pathways (Fig. 1.5). Physiologically, the MAPK/Erk pathway regulates cell proliferation, differentiation and apoptosis. The PI3K/Akt pathway controls cell proliferation, survival, the cell cycle, motility, as well as other cellular functions (35, 36). These pathways, usually coupled to the receptor tyrosine kinase (RTK) at the cell membrane, transduce extracellular growth stimuli that prompts downstream intracellular signaling (Fig. 1.5). Several oncogenic mechanisms which affect the signaling effectors in these pathways, have been identified in PTC (6).

The stimulation of the MAPK/Erk and PI3K/Akt pathways is initiated by extracellular signals, which activate receptor tyrosine kinases in the cell membrane. For the activation of the MAPK/Erk pathway, this leads to a cascade of activations, starting with RAS, then RAF (shown as BRAF), MEK, and Erk. Following its activation by phosphorylation, Erk enters the nucleus where it upregulates tumour-promoting genes and downregulates tumour suppressor genes (6)

For signaling through the PI3K/Akt pathway, receptor tyrosine kinase activation leads to the stimulation of RAS followed by PI3K. Activated PI3K

catalyses the conversion of phosphatidylinositol (4, 5)-bisphosphate (PIP2) to phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3). PIP3 activates 3‑phosphoinositide-dependent protein kinase 1 (PDK1), which consequently associates with Akt, leading to its phosphorylation and activation. On entry into the nucleus, activated Akt induces the expression of tumour-promoting genes. In the cytoplasm, activated Akt also activates other signaling molecules or pathways, a prominent one being the mTOR pathway, which has an important role in tumorigenesis (6).

Point mutation in the *BRAF* and *RAS* genes as well as chromosomal rearrangement involving the *RET* and *NTRK* genes are known deregulators of the MAPK/Erk pathway in PTC. These four mutually exclusive genetic lesions account for over 70% of PTC cases (37). In spite of their shared capacity for MAPK/Erk pathway activation, each type of mutation is associated with distinct histologic, clinical and biological features in PTC (Table 1.4) (38). As for aberrant activation of the PI3K pathway in PTC, mutations in the *RAS* as well as the *PIK3CA* and *PTEN* genes are well known causes. However, *PIK3CA* and *PTEN* mutations are rare, only found in 2-5% of PTC cases (39).

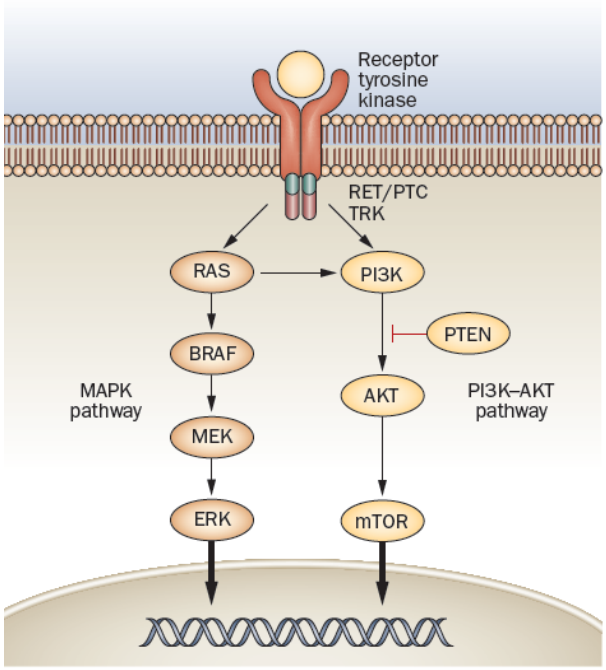

Figure 1.5: The main signaling pathways involved in thyroid carcinogenesis. The MAPK/Erk and PI3K/Akt pathways. Image was taken from Ref. 6.

TABLE 1.4: Specific genetic alterations and their characteristic features in PTC (taken from Ref. 38)

	BRAF	RET/PTC	RAS
Age	Older	Younger	Average
Histopathologic variant	Classic papillary Tall cell variant	Classic papillary Diffuse sclerosing	Follicular variant
Nuclear features	Pronounced	Pronounced	Less pronounced
Psammoma bodies	Common	Very common	Rare
Extrathyroidal extension	More common	Rare	Rare
Lymph node metastasis	Common	Very common	Rare
Tumor stage at presentation	More advanced	Early	Intermediate

1.4.1 BRAF Mutations

BRAF, a component of the MAPK/Erk pathway, is an isoform of a class of serine-threonine kinases. The most common *BRAF* gene mutation seen in PTC is the T to A transversion at nucleotide 1799, which results in a valine-to-glutamate substitution at amino acid residue 600 (V600E). BRAF (V600E) mutation occurs in approximately 45% of PTCs. Other rare *BRAF* mutations such as K601E point mutation, small in-frame insertions or deletions around codon 600 and AKAP9-BRAF rearrangement, which is common in PTC induced by radiation exposure has been reported (40, 41). All mutations lead to constitutive BRAF activation, resulting in continuous stimulation of the MAPK pathway and its downstream effectors. Observations from cell line and transgenic mice studies suggest a tumor-initiating role for *BRAF* mutation (42). However, additional studies showed that BRAF mutation also causes secondary genetic events with consequent activation of pathways like PI3K/Akt, and mTOR, which promote tumor progression (43, 44). Tumors with the BRAF mutation seem to exhibit aggressive features, like extrathyroidal extension, advanced stage at diagnosis, lymph node/distant metastases, and recurrence (38, 45). BRAF mutation also appears to be common in older patients (38).

1.4.2 RET/PTC Rearrangements

RET/PTC rearrangements are formed when the 3′ portion of the *RET* gene encoding its tyrosine kinase domain fuses with the 5′ portion of various genes (46). RET/PTC rearrangements are found in about 20% of PTC cases and are very common in individuals exposed to ionizing radiation and in pediatric patients (47, 48). So far, up to 16 types of RET/PTC arrangements have been discovered, but RET/PTC1 and RET/PTC3 are the most common ones in PTC. The fusion partners in RET/PTC1 and RET/PTC3, are *H4* or *NCOA4*, respectively (49, 50).

RET is not expressed in normal thyroid follicular cells. Upon rearrangement, the portion of *RET* coding for the tyrosine kinase domain is fused in frame with an active promoter of the fusion partner gene. As a result, the resulting chimeric RET receptor becomes constitutively expressed, lacks its extracellular and transmembrane domain such that it becomes resident in the cytosol and no longer requires ligand interaction for its activation (47, 51). Consequently, RET/PTC interacts with specific adaptor proteins to promote continuous activation of the MAPK/Erk pathway (52). Signal transducers and activators of transcription-3 (STAT3) pathway, a known oncogenic factor, are also known to be activated in RET/PTC rearrangements (53). RET/PTC mutations typically present at younger ages, have a classic histology and commonly spread to lymph nodes (38).

1.4.3 RAS Mutations

RAS genes encode the membrane G-proteins, which play important roles in cellular signal transduction processes. Point mutations in these genes are found in about 10 % of PTC cases (54). The mutations occur on codons 12, 13, and 61 of any of the three *RAS* isoforms namely NRAS, HRAS, and KRAS. In PTCs, the most common RAS mutation is seen on codon 61 of NRAS (54). The mutation maintains the RAS protein in its active, guanosine triphosphate (GTP)-bound conformation, resulting in chronic stimulation of several oncogenic pathways, most importantly the MAPK/Erk as well as the PI3K/Akt and STAT3 pathways (54). This mutation is associated with a lower rate of lymph node metastases (38, 54).

1.4.4 TRK Rearrangements

TRK rearrangements involving the *NTRK1* gene are the least prevalent mutations capable of activating the MAPK/Erk pathway. The *NTRK1* gene, located on chromosome 1q22, encodes a receptor tyrosine kinase involved in the regulation of growth, differentiation, and survival of neurons in the peripheral and central nervous system (55, 56). Like the *RET* gene, *NTRK1* is not expressed in normal thyroid follicular cells. TRK rearrangements juxtapose the intracellular tyrosine kinase domain of the *NTRK1* gene to the 5' end of other genes (55). There are 3 known types of TRK rearrangements in which *NTRK1* fuses with genes that are highly expressed in the thyroid follicular cells (57). Two of the fusion partners *TPM3* and *TPR* are also located on chromosome 1q; therefore these fusions are

intrachromosomal inversions (58, 59). The third rearrangement is a translocation because the partner gene *TFG* is resident on chromosome 3 (55). Nevertheless, all rearrangements result in constitutive expression and ligand-independent activation of the tyrosine kinase domain of NTRK1 (56). TRK rearrangements occur in less than 10% of papillary carcinomas and all three fusion types are found with approximately similar incidence (57, 60). Approximately 5% of radiation-induced PTC are positive for this rearrangement, especially the NTRK1/TPM3 type (61).

As summarized above, PTC is majorly MAPK/Erk-driven (mainly due to the BRAF-V600E mutations). However, it is noteworthy that a recent exploration of the genetic profile of 496 patient tumors provided a deeper pathological insight with implications for patient management (62). Their results suggest that BRAF (V600E) positive PTCs should not be considered a homogenous group, as it comprises about four molecular subgroups with variable degrees of differentiation (62). The differentiation status of papillary thyroid tumors impacts clinically relevant behaviors like RAI avidity which is associated with tumor aggressiveness and metastatic spread (63). This observation (62) further underscores the need for clearer molecular classifications that better reflects the underlying pathology in papillary thyroid tumors. Especially for the dedifferentiated, aggressive PTC variants, this may yield platforms for curative therapy beyond the blanket approach, which combines thyroidectomy and adjuvant RAI therapy.

1.5 Clinical Challenges on PTC Management

1.5.1 Nodal Metastasis

PTC has a great propensity for early invasion and regional lymph node metastasis (28, 29). Invasion of the extrathyroidal soft tissues of the neck is present in approximately 15% of cases (27). Clinically detectable lymph node infiltration occurs in up to 30% of PTC cases, while occult lymph node metastasis is as high as 90% (64). Also, 50% of papillary thyroid microcarcinomas (which are lesions 10 mm or less in diameter) harbor nodal metastases (64). Nodal metastases are associated with increased risk of local recurrence and distant metastases, which significantly impairs quality of life and is linked to poorer prognosis in patients >45 years of age (64-68). The presence of metastatic nodes at the time of initial examination increases the risk of developing nodal recurrence by 10 folds (64). Residual metastatic lymph nodes that remain after initial therapy are the most common cause of subsequent recurrence (64).

Age, sex, tumor size, and histopathologic properties of the tumor are among the most studied parameters for predicting lymph node involvement in papillary thyroid tumors. Patients younger than 20 years and older than 45 years, as well as those who are males, are more likely to have lymph node metastasis. Tumor size may not be an independent predictor of nodal spread as the data in the literature is contradictory, precluding any clear statement (63). Histological characteristics with possible predictive implication for nodal spread are vascular invasion and extracapsular involvement (63). For consistency in decision making, the use of these

clinical and pathological prognosticators for risk stratification has been standardized through a staging calculator named MASICS (69). These clinicopathologic parameters are unable to effectively predict nodal metastasis in papillary microcarcinomas (64). Investigative attempts to identify molecular predictors of nodal metastases have implicated cell cycle regulators (cyclin D1, p27), angiogenic proteins (VEGF-C), and metalloproteinases (MMP-2/9), but none is currently in clinical use (70, 71). Several oncogenes are now being assessed histologically in PTC including p53 and BRAF (63). BRAF V600E mutation is predictive of poor prognosis, but its role in predicting nodal spread remains controversial (72).

Therapeutic lymph node removal is usually performed when nodes are suspicious or proven to have metastatic disease based on pre- or intraoperative palpation, imaging, or cytological examination (63). However, many surgeons routinely perform prophylactic central neck lymph node dissections (CND) in patients without clinical or radiographic evidence of lymph node involvement. The advocates of prophylactic CND argue that the incidence of central neck metastases is high and the sensitivity of preoperative ultrasound is low (73). This prophylactic intervention is believed to reduce the possibility of nodal recurrence. However, CND is not without morbidity - the parathyroid glands and the recurrent laryngeal nerves, which are situated within this compartment, may be damaged. When performing a thyroidectomy with CND, compared to thyroidectomy alone, the added dissection potentially increases the possibility of hypoparathyroidism or recurrent nerve injury (73). Consequently, surgeons are faced with the quandary of either under-treating

patients, by avoiding lymph node dissection, or over-treating with complete removal of nodes, and possibly causing lifetime complications.

1.5.2 Resistance to RAI Therapy

Although most PTC patients are rendered free of disease following surgery and adjuvant RAI therapy, approximately 10-20% of them will recur with RAI-refractory metastatic disease. That is, the iodide-concentrating ability of the primary tumors and their metastatic deposits become suboptimal, rendering the treatment ineffective and creating poorer prognosis (74, 75). When this occurs, follicular cells are believed to have undergone dedifferentiation (loss of thyroid differentiation markers such as TSHR, TG, TPO, and NIS), as part of the malignant transformation and progression. Iodide trapping is a mechanism regulated by the TSH/TSHR system and the key machinery responsible is NIS. Downregulated expression of NIS has been observed in RAI-refractory thyroid tumors (76).

The ability of oncogenes to disrupt cell differentiation ultimately depends on the way by which they interfere with the transcription of the differentiation markers. Transformation of thyroid cells with RAS mutation is associated with the loss of TG and TPO expression, as well as reduced TSH-dependent functions such as iodide uptake (77). Although the mechanism of RAS-mediated dedifferentiation is not totally understood, it may occur in part by interference with the expression and/or activation of thyroid-specific transcription factors. Impaired TTF1 and PAX8 function have also been observed in RAS-transformed thyroid cells (77, 78). There

have been attempts to enhance NIS expression in order to re-establish radio-iodide uptake by utilizing agents such as retinoic acids and NIS gene transfection, but the results are not promising (79-81).

Management of RAI-refractory PTC remains challenging to clinicians, leading to a pattern of multiple and repeated RAI administration even when it may not have been beneficial (82). A patient is defined as having RAI-refractory disease if there is at least one lesion without RAI uptake or a lesion has progressed within a year following RAI treatment or persisted after the administration of a cumulative activity of more than 600mCi. High cumulative RAI doses (> 600 mCi) are now associated with a number of unfavorable side effects, including pulmonary fibrosis, xerostomia, lacrimal complications (84, 85) as well as an increased risk of secondary malignancies like leukemia (86). When RAI becomes ineffective, five-year survival is < 50% and 10-year survival is < 10% (83). Given the increased awareness of the adverse consequences of RAI resistance in PTC patients, the need for therapeutics with better efficacy and less toxicity is pressing.

Treatment modalities currently in use for RAI-refractory PTC include surgery, external beam radiation therapy (EBRT), percutaneous ethanol injection therapy (PEIT), and systemic therapy (87). Systemic therapies have become the only recourse for advanced, unresectable, RAI-refractory metastatic tumors that are not responsive to EBRT. Based on a study published in 1974, doxorubicin a traditional systemic chemotherapy, became the approved standard of care (83). Subsequent studies on doxorubicin have revealed poor response data and significant toxicity in patients (88).

Accordingly, a newer systemic approach driven by improved understanding of the oncogenic pathways driving thyroid cancer development and progression is now being explored. Using this targeted approach allows for specific blockade of aberrantly activated pathways, such that there is insignificant collateral damage to the hematopoietic, gastrointestinal and other body systems usually affected by standard chemotherapy (89).

Oncogenic alterations of tyrosine kinase members of the MAPK/Erk and PI3K/Akt pathways are known to result in amplified mitogenic, anti-apoptotic and angiogenic signals, thus creating an environment conducive for disease progression. For instance, *BRAF* mutations which drive constitutive activation of the MAPK/Akt pathway, have been associated with RAI resistance in papillary thyroid tumors (92). Similarly, owing to *RET* mutations, upregulated MAPK/Erk signaling was shown to inhibit apoptosis and protect PTC cells from doxorubicin-induced cytotoxicity (90, 91). Given the involvement of multiple tyrosine kinases in PTC progression, multitargeted tyrosine kinase inhibitors (TKI) are being investigated for the treatment of RAI-refractory PTC. These small molecular weight agents have been used with successful outcomes in various tumor types, including gastrointestinal stromal tumors, non-small-cell lung cancer, renal cell carcinoma, hepatocellular carcinoma and melanoma (93).

1.6 Tyrosine Kinase Inhibitors (TKIs)

Mostly as investigational therapies (used empirically), TKIs have become a major part of the tertiary and quaternary care given to patients with progressive papillary thyroid tumors refractory to RAI (94-100). Sorafenib and lenvatinib are the oral multi-TKIs already approved by the U.S. Food and Drug Administration (FDA) for treating RAI-refractory PTC (96, 100). Kinases affected by sorafenib include BRAF, vascular endothelial growth factor receptors (VEGFRs) 1 - 3, platelet-derived growth factor receptorβ (PDGFRβ), RET, and c-kit (96, 97). Clinical evidence suggests that sorafenib blocks thyroid cancer growth through both antiproliferative and antiangiogenic mechanisms (96, 97). Its approval was based on a Phase 3 trial in patients with RAI-refractor locally advanced or metastatic DTC which showed a 5-month improvement in median progression-free survival (96). Recently approved, lenvatinib has the capacity to inhibit VEGFR 1 - 3, fibroblast growth factor receptors (FGFRs) 1–4, RET, c-KIT, and PDGFRs (98-100). In a Phase 3 study, lenvatinib treatment yielded significant improvements in progression-free survival and the response rate among patients with progressive DTC resistant to RAI (100). The primary endpoint of this study was progression-free survival and the median was 18.3 months in the lenvatinib group compared to 3.6 months in the placebo-treated. The response rate was 64.8% in the lenvatinib group (with 4 complete responses and 165 partial ones) versus 1.5% in the placebo-treated (100).

Though the efficacy of these TKIs is impressive, huge safety concerns still remain based on the clinical study results (96, 100). In the SELECT trial, patients

who received lenvatinib had more adverse effects at 97%, compared to 60% in the placebo group. Adverse effects associated with lenvatinib treatment include hypertension, proteinuria, arterial thromboembolic effects, venous thromboembolic effects, renal failure, hepatic failure and gastrointestinal fistula. Also, 6 out of the 20 deaths that occurred within the lenvatinib group were considered drug-related (100). Common adverse outcomes observed with sorafenib treatment include hand–foot skin reaction (76·3%), diarrhoea (68·6%), alopecia (67·1%), and desquamation (50·2%) (99). These adverse effects and toxicity led to frequent dose interruption, and reduction during the clinical trials (96, 100).

An expanding repertoire of TKIs for RAI-Refractory PTC patients are still under clinical trials. They have been shown to produce progression-free survival of approximately one year (range: 7.7-19.6 months) and partial response rates of up to 50% (by Response Evaluation Criteria in Solid Tumors), with majority of patients experiencing tumor shrinkage (101). Though they have potential clinical value, guidance on selection and use is still required given the varied outcomes and adverse side effects which accompany their use (101). These shortcomings underscore the pressing need to identify molecules that drive PTC metastasis, which could be targeted for safer and more effective therapy. Datasets recently acquired by our lab suggest that platelet-derived growth factor receptor α, a tyrosine kinase receptor, may be driving metastasis in PTC (102).

1.7 Platelet-Derived Growth Factor (PDGF) ligands and Receptors

There are four PDGFs (PDGF-A, PDGF-B, PDGF-C and PDGF-D) encoded by separate genes located on chromosomes 7, 22, 4 and 11, respectively (103). They exist as five disulphide-bonded functional homo- or heterodimer (PDGF-AA, -BB, -CC, -DD and -AB). The A- and B- chains are synthesized as inactive precursors, which are cleaved in the producer cell during secretion, and released into the extracellular milieu as active forms. On the other hand, the C- and D- chains are secreted as inactive forms containing N-terminal CUB domains, and have to be enzymatically cleaved to allow binding to their receptors (104). This cleavage is an important regulatory step performed by tissue-type plasminogen activator or plasmin in PDGF-CC, and by urokinase-type PA or matriptase in the case of PDGF-DD (104).

The PDGF ligands mediate their cellular effects through structurally similar receptors PDGFRα (170KDa) and PDGFRβ (180KDa), which belong to the class III receptor tyrosine kinase family. These 2 transmembrane receptors have five extracellular Ig-like domains for ligands binding, and an intracellular tyrosine kinase domain, which has a characteristic inserted sequence of about 100-amino acid residues without similarity to known kinase domains. The five dimeric PDGF ligands have distinct binding patterns and affinities for the receptors (Fig. 1.6). All ligands except PDGF-DD induce αα homo-dimerization, whereas only PDGF-BB and -DD activate ββ homodimers. All ligands except PDGFAA will induce heterodimers αβ in cells co-expressing both receptor types (104).

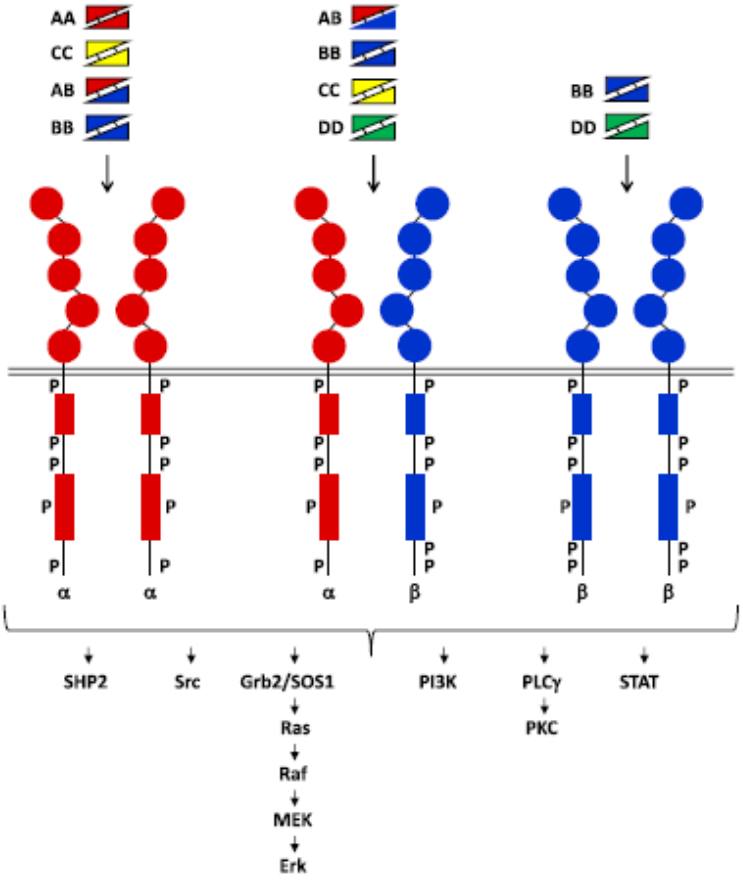

Figure 1.6: PDGF isoforms, receptor binding and signaling cascade. The dimeric PDGF ligands (red, blue, yellow and green) have specific binding patterns and affinities for the receptors. The extracellular parts of the receptors contain 5 Ig-like domains, ligands bind preferentially at domains 2 and 3, while domain 4 stabilizes the resulting receptor dimer. Ligand binding results in 3 receptor-dimer configurations – αα, αβ, and ββ. The intracellular parts of the receptors contain tyrosine kinase domains split into two parts by an intervening sequence. P denotes the tyrosine residues which are autophosphorylation sites outside the kinase domain. Ligand-induced dimerization induces autophosphorylation of the receptors, which activates their kinases and create docking sites for SH2-domain-containing signaling molecules (including SHP2, Src, PI3K, PLCγ, and STAT). Image was taken from Ref. 106.

1.7.1 PDGF Signaling Cascade

Ligand binding occurs mainly to Ig-like domains 2 and 3, and causes dimerization of the receptors, which is further stabilized by direct receptor-receptor interactions involving Ig-like domain 4 (Fig. 1.6). Dimerization brings the intracellular parts of the receptors close to each other promoting trans-autophosphorylation between the receptors. Phosphorylation of a tyrosine residue in the activation loop of the kinase, in addition to other specific tyrosine residues in the cytoplasmic regions of the receptors enhances the catalytic activity of the kinase. The phosphorylated tyrosine residues also provide docking sites for downstream SH2-domain-containing signaling molecules (104). Documented interactions between specific phosphorylated tyrosine residues and different SH2-domain-containing signaling molecules are shown in Fig. 1.7. At least, 11 and 12 autophosphorylation sites have been identified in the α- and β-receptors, respectively (105, 106).

The phosphorylated tyrosine residues on these sites are known to selectively bind about 10 different types of SH2-domain-containing molecules. As seen in Fig. 1.7, these include signaling molecules with intrinsic enzymatic activities, such as tyrosine kinases of the Src family, PI3K, SHP-2 tyrosine phosphatase, and phospholipase C-γ. The activated receptors can also bind and activate signal transducers and activators of transcription (STATs), which subsequently translocate to the nucleus where they act as transcription factors (105, 106). SH2-domain-containing adaptor molecules which lack intrinsic enzymatic activities can also interact with the activated receptors, to form bridges between the receptors

and other signaling molecules (105, 106). For instance, an adaptor protein Grb2 interacts with activated PDGFR, binds the nucleotide exchange molecule SOS1, and activates the MAPK/Erk pathway (Fig 1.6). As seen in Fig. 1.7, PDGFRs bind other adaptors, e.g. Shc, Nck, Crk and GAP, which mediate interactions with a plethora of different downstream signaling molecules (104-106).

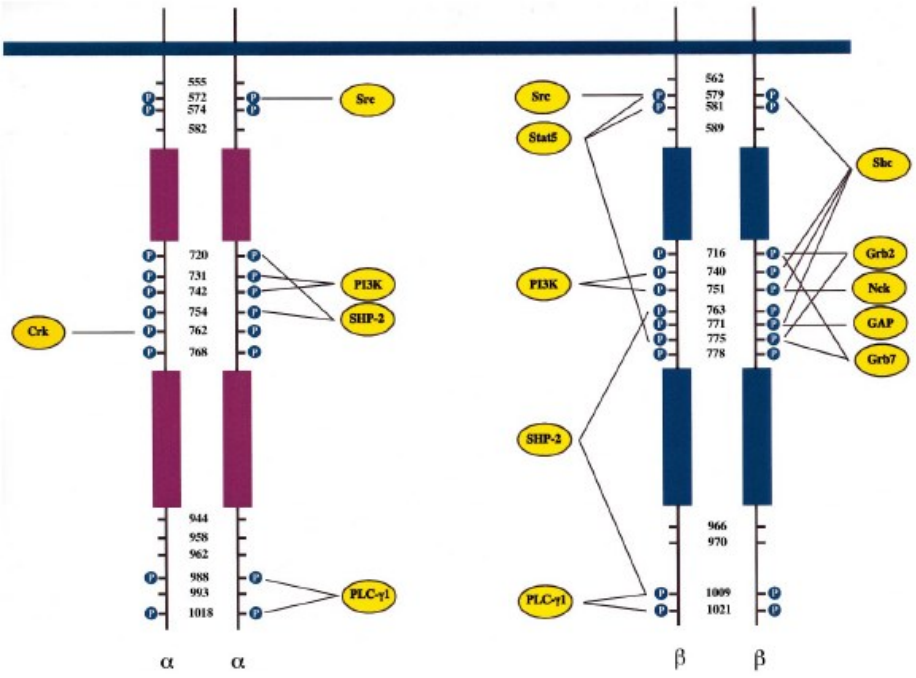

Figure 1.7: Interaction between PDGFR-α and -β receptors and SH2-domain containing signal transduction molecules. The intracellular part of homodimeric complexes of α and β receptors are depicted. All tyrosine residues outside the catalytic domains and their numbers are indicated; known autophosphorylation sites are indicated by an encircled P. The conserved tyrosine residues in the kinase domains which are involved in the regulation of the catalytic activities are also indicated. The known interactions between individual phosphorylated tyrosine residues and different SH2 domain containing signal transduction molecules are shown. Image was taken from Ref. 104.

1.7.2 PDGF ligands and Receptors in Normal Physiology

PDGF stimulates cell proliferation and migration in a number of physiological processes (107). Insights into the physiological implication of PDGF signaling emanated largely from studies using mice deficient in PDGF ligands or their receptors (123). Particularly, the critical roles of PDGFs and PDGFRs in embryo development have been revealed. During embryonic development, PDGF ligands are usually secreted by epithelial or endothelial cells in various organs (106). They act in a paracrine fashion on neighboring mesenchymal cells like fibroblasts, pericytes and smooth muscle cells, which express the PDGF receptors. Signaling via PDGFRα is important for the development of the facial skeleton, hair follicles, spermatogenesis oligodendrocytes astrocytes, lungs and intestinal villi. PDGFRβ signaling is important for the formation of blood vessels, kidneys and white adipocytes (106).

Mice lacking PDGFRβ died shortly after birth - they were severely deficient in vascular smooth muscle cells, such as vessel pericytes and mesiangial cells of kidney glomeruli (123). PDGFRα knock-out mice had a more severe phenotype, with defects in multiple organs such as lung, skeleton, testis and the central nervous system (123). PDGFRα-null embryos did not survive past embryonic day 15, with most dying by day 11.5 (123). It was suggested that the striking difference between the PDGFRα- and PDGFRβ-deficient mice may be related to the different spatiotemporal expression patterns of these receptors. To assess whether this observation was a result of different signaling paths, the intracellular domain of PDGFRα was replaced with that of PDGFRβ in knock-in mice, and vice versa (123).

Results from these experiments showed a large overlap in the signaling capabilities of the two receptors, although the PDGFRα cytosolic domain only partially rescues PDGFRβ signaling (123).

Similarly, PDGFRα and PDGFRβ transduce overlapping but discrete biological signals in vitro (107). When both receptors coexist in the same cell, separating the signaling pathways and biological responses associated with the heterodimeric receptors from those of the homodimers has proven difficult (107). The extensive cross-talk between the different signaling pathways (Fig. 1.6) makes it challenging to assign individual pathways to specific responses. Most of the existing functional knowledge of the receptors in cells was obtained from exploring the αα and ββ homodimers (Fig. 1.7). Accumulating evidence have shown that in a cell-type- and context-dependent manner, several signaling pathways contribute to each of the cellular responses which includes proliferation and migration (106).

In adults, PDGF promotes wound healing and regulates the interstitial fluid pressure in tissues (108). The limited roles of the PDGFRs in adults, compared to embryos, allow for reasonably safe use of their inhibitors as therapeutics for diseases in which they have pathological roles. For instance, cancer patients who receive a long term treatment with imatinib (a potent inhibitor of the PDGFRs), had no obvious healing difficulty. The only side effect ascribed to PDGFR inhibition in these patients was edema, which could be linked to compromised interstitial fluid pressure regulation (123).

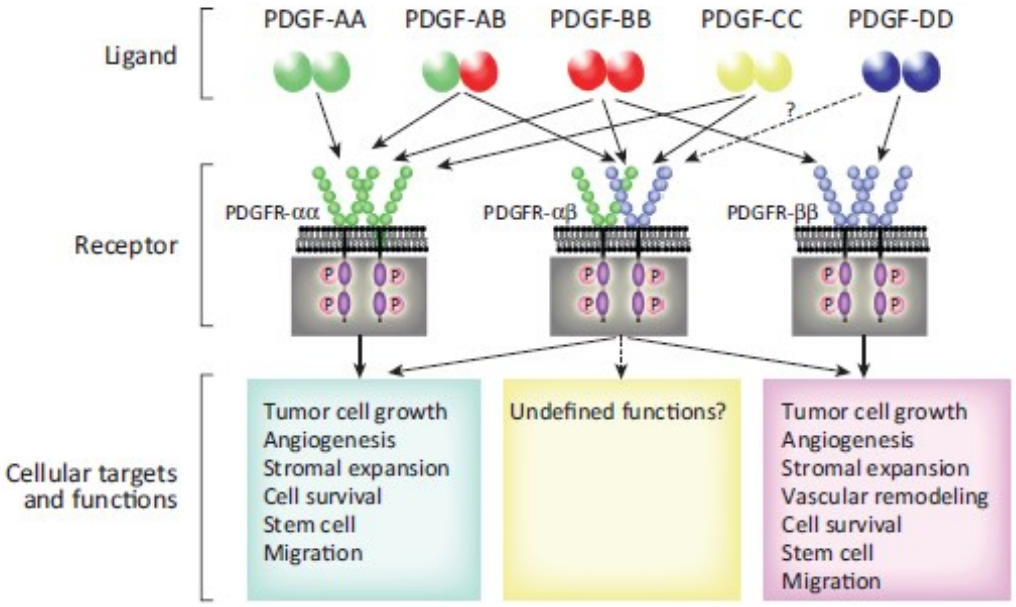

Figure 1.8: PDGF-PDGFR signaling and functions. The interaction between PDGF-DD and αβ heterodimers is yet to be clearly defined. When activated, PDGFRs transduce overlapping but distinct biological signals. For example, PDGFRβ but not PDGFRα is involved in vascular remodeling, maturation, and stability. Image was taken from Ref. 107.

1.7.3 PDGFR signaling in Cancer

Overwhelming evidence highlighting aberrant PDGFR signaling as a driver of malignant transformations in various cancers continues to emerge (109). Genetic alterations leading to constitutive activation and autocrine stimulation of PDGF receptors have been identified in numerous malignancies. These include chromosomal translocations producing fusion to functionally unrelated genes, activating mutations, and gene amplification. Furthermore, it is believed that epithelial tumor cells undergo epithelial-mesenchymal transition (EMT) which enhances their invasiveness and metastasis (110).

Autocrine PDGFR signaling pathways have been shown to promote EMT in several cancer types, including breast (111) and prostate (112). During the EMT process, epithelial tumor cells overexpress the PDGFRs. Therefore, they become responsive to the PDGFs which they would otherwise be insensitive to as normal epithelial cell (which have little or no expression of the receptors) (111). Evidence for paracrine stimulation of fibroblasts and perivascular cells by PDGF ligands secreted by neighboring epithelial tumor cells also exist (103). In some solid tumors, the non-malignant stromal compartment constitutes most of the tumor mass in which cancer associated fibroblasts (CAFs) are a major component (107). Xenograft growth observed in a recent study where implanted melanoma cells did not express the PDGFRs, suggested a paracrine mechanism. It was shown that PDGF supplied by the tumor cells interacted with PDGFRα-expressing CAFs to induce tumor growth. PDGF-dependent pericytes have been shown to enhance tumor angiogenesis (113).

PDGFRα has been identified as the key receptor in many of the PDGF mediated tumorigenic processes. Tumors with aberrant PDGFRα signaling include ovarian, prostate, breast, lung, renal cell, glioma, melanoma, gastrointestinal stromal, and bone cancer (103). About 5% of gastrointestinal stromal tumors harbor point mutations in the PDGFRα gene, leading to amino acid residue replacements in critical regions of the receptor causing activation of the kinase (114). Similarly, in about 10% of glioblastoma multiforme cases, gene amplification results in augmented expression of the alpha receptor takes place (115). Amplification of PDGFRα has also been observed in oligodendrogliomas, esophageal squamous cell carcinoma, and artery intimal sarcomas (106). The amplification increases the sensitivity of cells to minute levels of PDGF ligands, or if the number of receptors is high enough, signaling may proceed in a PDGF-independent manner (106). An activating deletion mutation in the PDGFRα gene has also been detected in a human glioblastoma (116). PDGFRα overactivity is also associated with metastasis and disease progression (117, 118). In rhabdomyosarcoma patients, upregulated expression of PDGFRα has been linked with poor prognosis (106). A recent study showed that PDGFRα promotes invadopodia formation as well as other metastatic phenotypes in response to EMT-inducing signals in breast cancer cells (119). Invadopodia are actin-rich membrane protrusions that promote extracellular matrix degradation and invasiveness in metastatic tumors and transformed cells.

Given the consequences of its aberrant signaling highlighted above in various tumor types, PDGFRα is fast becoming an attractive target for newly-developed

TKIs. For instance, Imatinib a TKI capable of blocking the activity of PDGFRα (as well as ABL kinase and KIT) has become an FDA approved treatment for gastrointestinal stromal tumors (120). Crenolanib, which inhibits signaling of wild-type and mutant PDGFRα with high potency is also being evaluated for the treatment of PDGFRα-mutant gastrointestinal stromal tumors, PDGFRα-amplified gliomas and non-small-cell lung cancers (NSCLC) (120-122). Crenolanib suppressed the proliferation and migration in NSCLC cells, and its antitumor activity was further confirmed in Xenograft models (121).

1.8 HYPOTHESIS AND OBJECTIVES

1.8.1 Rationale

The routine treatment for PTC patients in the United Sates and several European countries, combines surgical resection with RAI ablation of remnant thyroid and metastatic deposits (124). Although with this regimen prognosis is generally encouraging, the high propensity of papillary thyroid tumors for lymph-node metastasis increases the risk of recurrence, necessitating repetitive surgery (125, 126). Recurrent PTC is associated with increased morbidity and mortality with significant psychoeconomic consequences (124). Overall, the recurrence rate of PTC is high, around 20–30% at 15–20 years (124). Compartment-based removal of neck lymph nodes in appropriate patients is effective in preventing the recurrence of PTC, but the selection of patients who will benefit from lymph node dissection remains a challenge. Preoperative ultrasonography cannot predict metastatic risk, and the extent of the initial surgery is usually left to the physician's discretion (127). Clearly, accurate identification of tumors with high metastatic potentials is needed to inform surgical interventions. This will minimize recurrence and repetitive surgery in high-risk patients.

Another risk associated with the nodal spread of PTC is tumor resistance to RAI therapy. The recurrent disease often progresses to become surgically inoperable and resistant to RAI ablation (128, 129). Metastatic PTC cells usually become de-differentiated, disrupting the function of the NIS, leading to reduced or failed ability

to concentrate therapeutic levels of radioiodide. About one-third of metastatic differentiated thyroid cancers (of which majority are PTCs) become refractory to RAI ablation (129). Higher (or repeated) doses are often administered in these cases, but evidence suggesting that RAI is of little benefit in patients with RAI-refractory disease has been reported (128, 129). As such, the management of RAI-refractory metastatic PTC is challenging since RAI is generally not recommended in this group of patients. Thus, new therapeutic options are now being explored for this group of patients. Given that the tyrosine kinase activity of growth factor receptors is central to the activation of oncogenic pathways; intense interest is now in developing pharmacologic inhibitors of these kinases. TKIs are now considered for investigational use in quaternary care settings for patients with RAI-refractory PTC. However, due to the safety concerns, and mixed outcomes observed in patients, guidance on selection is still required. Systematic approaches to TKI selection have been prevented by lack of comprehensive understanding of the pathologic consequences of the aberrant tyrosine kinase signaling in metastatic PTCs (101). Specific targeting of identified aberrant tyrosine kinases will likely provide less toxic, but more effective and reproducible outcomes in patients.

To expand prognostic and treatment options for patients with RAI-refractory metastatic PTC, molecular techniques are needed to aid the identification of aggressive variants and to determine the pathways responsible for metastatic spread and loss of NIS function. In our laboratory, Zhang *et al.,* (102) recently identified a strong positive association between nodal metastasis and PDGFRα expression in PTC

tumors. Biopsies from metastatic primary tumors exhibited high levels of PDGFRα, while primary tumors without metastases showed minimum PDGFRα staining (102). This is compelling evidence that PDGFRα signaling is involved in the nodal spread of PTC. Consequently, in this study, we assessed the potential utility of PDGFRα as a novel metastatic risk identifier, and drug target in the management of metastatic PTC tumors, particularly those resistant to RAI ablation. PDGFRα staining may be a useful adjunct in the diagnostic assessment of papillary tumors, allowing surgeons and oncologists to accurately identify patients needing aggressive surgery or radioactive iodide, thus avoiding these costly treatments in patients with indolent tumors. We believe that therapies based on PDGFRα will not only provide increased effectiveness through direct killing of tumor cell and abrogating aggressiveness, but such blockade may also partially restore iodide uptake and reduce resistance to RAI therapy.

1.8.2 Hypothesis

PDGFRα expression is prognostic of lymphatic metastasis in PTC patients, its activation drives aggressiveness and promotes dedifferentiation, resulting in reduced iodide uptake in PTC cells, and blocking its activity will reduce the severity of these pro-metastatic phenotypes.

1.8.3 Specific Aims

1) To determine the prognostic value of PDGFRα and its associated ligands in patient samples of papillary thyroid cancer **(Chapter 3)**
   a) To survey the expression of PDGFRα and PDGF ligands in clinical samples with and without nodal metastasis.
   b) To correlate the expression of the PDGF/PDGFRα system with metastatic behavior in order to determine the potential of this system for metastatic risk prediction
2) To explore the phenotypic and signaling consequences of PDGFRα signaling in cellular models of PTC **(Chapter 4)**
   a) To analyze the functional status of PDGFRα, as it pertains to aggressive phenotypes like migration and functional invadopodia formation in cellular models of PTC.
   b) To examine the downstream signaling pathways responsible for the PDGFRα-induced phenotypes in PTC cells.
   c) To determine the extent to which the pharmacological blockade of PDGFRα signaling can downplay the aggressive features associated with it.
3) To explore the link between PDGFRα signaling, dedifferentiation and RAI therapy resistance in papillary thyroid cancer **(Chapter 5)**.
   a) To outline the mechanism for PDGFRα-induced dedifferentiation, and iodide uptake suppression in PTC cells.

b) To examine the effects of blocking PDGFRα activity via pharmacologic molecules, on the extent of differentiation, and the iodide concentrating capacities of PTC cells.

c) To correlate PDGFRα expression with RAI therapy resistance and disease recurrence in patient specimens.

1.9 References

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CHAPTER 2: MATERIALS AND METHODS

2.1 Patients and Tissues

Ethics approval was obtained through the University of Alberta Heath Research Ethics Board and informed consent was obtained from all patients. Two pathologists separately confirmed histological diagnoses of the primary tumors, and the presence of metastases in the nodes sectioned. The classification of thyroid tumors was based on the World Health Organization criteria (2004).

2.2 Immunohistochemistry (IHC) and Scoring

Immunohistochemical staining was performed using standard techniques. Briefly, TMAs were freed of paraffin in xylene and rehydrated through graded alcohols. Heat-induced antigen retrieval was performed by pressure-cooking slides in the microwave with citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was quenched by incubating slides in 3% H_2O_2 for 10 min, followed by treatment with avidin/biotin blocking reagent (Dako Burlington, ON, Canada) for 15 min at room temperature. Immuno-reactivity was then assessed by overnight incubation with primary antibodies at 4^oC in a humidified chamber. Slides were washed thrice with TBS and incubated with the biotinylated universal secondary antibody followed by streptavidin-HRP complex according to the manufacturer's instructions (LSAB+ system; Dako). For color development, tissue sections were incubated with 3, 3'-diaminobenzidine (Dako) and counterstained lightly in hematoxylin for 3 min.

All specimens had representative sections (more than 90%) of the clinical diagnosis, and scoring was performed by 2 pathologists who had no prior knowledge of the diagnosis.

2.3 Materials

PDGF ligands AA, BB and DD were purchased from Life Technologies (Grand Island, NY). Doxycycline hyclate was purchased from Sigma (St Louis, MO, USA). Crenolanib was obtained from Selleckchem (Houston, TX). MAPK/Erk inhibitor U0126 and PI3K/AKT inhibitor LY294002 were purchased from Cell Signaling Technology (Beverly, MA). STAT3 inhibitor, Stattic, and Quercetin, an inhibitor of β-catenin transcriptional activity were obtained from Sigma-Aldrich (Oakville, ON, Canada). Inhibitor stock solutions were prepared by reconstitution in dimethyl sulfoxide (DMSO; Sigma-Aldrich) following the suppliers' instructions. Dilutions were performed in growth media to make the working concentrations.

2.4 Cell Culture

Human PTC cell lines BCPAP, 8305C and KTC1 were purchased from American Type Culture Collection (Rockville, MD) and cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) under an atmosphere of 5% CO2 at 37 °C.

2.5 Isolation of Primary Cells

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 h 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 transferrin, 10 ng/mL somatostatin and 10 ng/mL glycyl-L-histidyl-L-lysine).

2.6 Stable Transfections

To stably express human PDGFRα complementary DNA in established and primary cells, we used a doxycycline-inducible retrovirus system (Lenti-X Lentiviral Expression Systems; Clontech Laboratories, Inc., Mountain View, CA, USA). These cells were first transduced with the LVX-Tet-On advanced lentivirus (Neo+) followed by selection in G418 (1 mg/ml). Resistant cells were then transduced with the LVX-Tight-Puro (Puro+) vector or sequence-verified derivatives expressing wild-type human PDGFRα cDNA, followed by selection in puromycin (2.5 μg/ml). cDNA expression was induced by addition of doxycycline (2 μg/ml) and protein expression was verified by immunoblotting for the total and phosphorylated forms of PDGFRα.

To selectively and stably knock down the expression of PDGFRα in 8305C cell lines we used the HuSH-29 shRNA Vector system (HuSH-29 shRNA Retroviral Vector Systems; OriGene Technologies, Inc.). Briefly, PTC cells were transduced

with the pRS shRNA retrovirus system (Puro+) followed by selection in puromycin (2.5 ug/mL). Resistant cells were assessed by western blot to select the sequences that produced the highest levels of protein expression knock-down. The transduced 8305C cells were further sorted by flow cytometry to select a strongly negative PDGFRα population. To stably knock down the PDGFRα receptor, cells were transduced with the pGFP-BR-S shRNA retrovirus system (BSD+) followed by selection in blasticidin (500ug/mL). Resistant cells were again assessed by western blot to select the sequences that produced the highest levels of protein expression knock-down.

2.7 Western Blotting

Cells were washed with phosphate-buffered saline (PBS), and cell proteins were solubilized using RIPA buffer supplemented with 0.1 mM phenylmethylsulfonyl-fluoride, protease inhibitor cocktail (1:100; Sigma-Aldrich) and phosphatase inhibitor cocktail 2 (1:50; Sigma-Aldrich). Lysates were centrifuged at 20,000 g for 15 min to remove cell debris. Supernatants were collected and protein concentrations were determined using BCA Protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL). Protein samples were boiled for 10 min in SDS sample buffer, resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA). Membranes were blocked for 1 h with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and subsequently incubated overnight at 4 °C with primary antibodies. Membranes were washed thrice with TBS-T and incubated for 1 h at room temperature in the appropriate secondary antibody

conjugated with horseradish peroxidise (Bio-Rad). Following three washes with TBS-T, protein bands were detected using a chemiluminescent detection kit (Pierce). Equal loading of protein was confirmed with either anti-GAPDH (sc-25778) or anti-γ-tubulin (GTU-88:T6557).

2.8 Three-Dimensional (3D) Cell Culture

Growth factor-reduced Matrigel (BD, Cambridge, MA), 130 µl was spread as a thick layer on 8-well chamber slides, and polymerized at 37 °C for 15 min. Then cells grown as monolayer were trypsinized, re-suspended in the appropriate growth media containing with 2% Matrigel and plated on top of the pre-coated Matrigel. Cells (6000) were seeded into each well and growth medium was replenished every two days until colonies were established.

In some experiments, colony development proceeded in the sustained presence of PDGFAA (50 ng/ml), LY294002 (10 µM), U0126 (10 µM), Stattic (2.5 µM), Quercetin (10 µM) or Crenolanib (1 µM). Cells were fixed at indicated times with 4% paraformaldehyde and phase-contrast images were acquired using an AMG EVOS digital inverted microscope. The degree of branching was assessed using these images and quantified with Adobe Photoshop.

2.9 Immunofluorescent Staining and Confocal Imaging

For F-actin staining, cells in 3D culture were 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.

To confirm PDGFRα-induced accumulation of nuclear pSTAT3, cells were seeded at 4,000 cells per well on 8-well chamber slides. The cells were serum-starved for 24 h and treated with or without PDGF-AA (50 ng/ml) for 20 min, fixed in 4% paraformaldehyde and permeabilized using ice-cold methanol.

Samples were then incubated overnight at 4°C with the appropriate primary antibodies (PDGFRα, 1:800 and pSTAT3, 1:50) and Alexa 488-conjugated phalloidin for F-actin staining (Life Technologies). For visualization of the PDGFRα and pSTAT3, cells were further incubated with Alexa 594- and Alexa 488- conjugated secondary antibodies (Life Technologies) respectively for 1 h. 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.

2.10 Invadopodia Assay and Fluorescent Visualization

For the invadopodia assay, the QCM™ Gelatin kit (EMD, Millipore) was purchased and used according to the manufacturer's guidance. Briefly, to facilitate attachment of the cy3-conjugated gelatin, 8-well glass chamber slides were first

coated with poly-L-lysine for 20 min, rinsed three times with PBS, incubated for 15min in glutaraldehyde and washed three times with PBS. Gelatin was mixed at a 1:5 ratio (fluorescently-labeled unlabeled gelatin), 200 μL of the mixture was incubated in each well for 10 min, followed by three rinses in PBS. Disinfection with 70% ethanol for 30 min, wells were rinsed with PBS, and free aldehydes were quenched by the addition growth medium for 30 min. Then, BCPAP-Empty and BCPAP-PDGFRα cells were seeded at 10, 000 cells/well and cultured for 4 days in the presence of PDGFAA (50 ng/ml) or Crenolanib (1 μM). Untreated cells without both treatments were also cultivated.

For immunofluorescent imaging of invadopodia activity (gelatin degradation), cells were fixed for 30 min at RT in 4% paraformaldehyde, rinsed twice with fluorescent staining buffer (PBS with 2% blocking serum and 0.25% Triton X-100 for cell permeabilization). To assess F-actin expression, cells were blocked in the fluorescent staining buffer for 1h, incubated with Alexa-488 conjugated phalloidin for 45 min, the cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 5 min and samples were mounted with Prolong Gold Anti-fade reagent (Life Technologies). Images of gelatin degradation and F-actin staining was taken Images were taken with a Zeiss LSM 710 Axio Observer inverted 34-channel confocal microscope 20X objective lens and analyzed with Zeiss Zen software. Metamorph software (Molecular Devices) was used to perform image analysis. Invadopodia were identified by areas of gelatin degradation characterized by loss of red fluorescence. Gelatin degradation was measured by quantifying the average area

of non-fluorescent pixels per field. 5 random fields were imaged per condition and each independent experiment was performed at least 3 times and averaged.

2.11 Taqman Low Density Assays (TLDAs)

BCPAP-Empty and BCPAP-PDGFRα cells were grown in growth factor-reduced Matrigel as described above, in the presence of PDGFAA (50 ng/ml). Thereafter, the structures formed were recovered from the Matrigel matrix using the Corning Cell Recovery Solution (Bedford, MA) according to manufacturer's instructions. Briefly, the medium was removed, the matrigel embedded structures were washed 3 times with cold PBS and the appropriate volume of the Recovery Solution was added. The structures/gel were scraped into tubes and left on ice for 1 h and spun down at 300 x g for 5 min at 4°C. Then, the released 3D structures were washed in ice-cold PBS twice, and processed for RNA extraction.

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturers' instructions. 2 μg of RNA was reverse transcribed in a 20 μl reaction volume. The resulting cDNA samples were analyzed on customized Taqman Low Density Arrays (TLDAs; Applied Biosystems). Each 384-well TLDA card contained two identical 192 gene-set. 188 genes encode proteins relevant in mechanisms which drive tumor metastasis while 4 replicates of the GAPDH gene were included to normalize the expression of the target genes. Each 2 μL well contains specific primers

and probes, capable of detecting a single gene. Appropriate amounts of the cDNA and the TaqMan Universal Master Mix (Applied Biosystems) were combined and loaded onto the TLDA wells as advised by the manufacturer. BCPAP-Empty and BCPAP-PDGFRα cells were compared on the same TLDA cards in three independent experiments.

qPCR analyses were performed according to the manufacturer's instructions using the 7900HT fast real-time PCR system (Applied Biosystems). The Comparative threshold (Ct) values were estimated by the SDS2.3 software package (Applied Biosystems) and exported to RQ-manager v1.2 (Applied Biosystems) for relative quantitation. Ct values greater than 35 were designated "not-expressed" and excluded from the analyses. BCPAP-Empty cells were used as the calibrator, for which all gene expression values were assigned a relative value of 1.00.

2.12 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 (1, 2). 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 for assay validation and as a 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 h at 37°C, 5% CO_2 atmosphere and 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 min at RT, and absorbance readings (420 nm) were taken.

2.13 Trypan Blue Exclusion and MTS assays

To assess the effect of LY294002, U0126, Stattic, Quercetin, or Crenolanib on proliferation, cells were seeded at 35,000 cells per well in 12-well culture plates. After 24 h of treatment, cells were trypsinized, stained with trypan blue (Sigma) and counted with a hemocytometer. Results are expressed as total numbers of viable cells. The MTS assay (Promega, Madison, WI, USA) was also performed to assess the cytotoxicity of Crenolanib at various concentrations after 5 days of treatment. Results are expressed as a percentage of untreated controls.

2.14 Cell Migration Assay

Cell migration was assessed using 96-well Boyden Chamber apparatus (3). Cells were serum-starved overnight and detached with PBS containing 2 mM EDTA and 0.1% BSA, pH 7.4. Cells were washed three times with serum-free DMEM and suspended in DMEM containing 0.1% BSA to achieve a concentration of 300,000

cells/ml. The top chambers were loaded with 100 µl of cell suspension, and the bottom chambers were filled with DMEM containing the agonist (50 ng/ml PDGF AA). The top and bottom chambers were separated with polycarbonate membrane containing 8 µm pores, which was coated with 10 µg/ml fibronectin overnight at 4°C. Cell migration was measured over 4 h at 37°C. Cells attached to the membrane were fixed with methanol. Then, cells on the top surface of the membrane were wiped off while those that migrated across the membrane to the bottom were stained with crystal violet and quantified with an Odyssey infrared imaging system (LI-COR Biosciences).

2.15 Antibodies

Information about the primary antibodies used in Chapters 3, 4, and 5 are shown in **Tables 2.1, 2.2 and 2.3.**

Table 2.1: Primary antibody information (Chapter 3)

Antibody	Catalogue number	Dilutions	
		IHC	Flow cytometry
PDGF-AA	Santa Cruz (sc-128)	1:75	
PDGF-BB	Santa Cruz (sc-127)	1:75	
PDGF-DD	Santa Cruz (sc- 137030)	1:75	
PDGFRα	Cell Signaling (5241)	1:50	
PDGFRα-PE	Cell Signaling (8533)		1:50

Table 2.2: Primary antibody information (Chapter 4)

Protein	Catalogue Number	Dilution
Phospho-Akt	Cell Signaling (4051)	1:1000
Akt	Cell Signaling (9272)	1:2000
N-Cadherin	Cell Signaling (4061)	1:1000
Phospho-Erk	Cell Signaling (9106)	1:1000
Erk	Santa Cruz (sc-94)	1:2000
Phospho-GSK3β (S9)	Cell Signaling (9336)	1:1000
Phospho-PDGFRα (Y754)	Cell Signaling (2992)	1:1000
PDGFRα	Cell Signaling (5241)	1:1000
Phospho-PDGFRβ (Y751)	Cell Signaling (3166)	1:1000
PDGFRβ	Santa-Cruz (sc-80991)	1:1000
Phospho-PDGFRα/β (Y849/ Y857)	Cell Signaling (3166)	1:1000
Slug	Cell Signaling (9585)	1:1000
Snail	Cell Signaling (3879)	1:1000
Phospho-STAT3	Cell Signaling (9131)	1:1000
STAT3	Cell Signaling (9139)	1:1000
Tubulin	Sigma Aldrich (T6557)	1:10000
Twist-1	Santa Cruz (sc-81417)	1:250
Vimentin	Cell Signaling (5741)	1:1000

Table 2.3: Primary antibody information (Chapter 5)

Antibody	Catalogue number	Dilutions	
		Blotting	IHC
PDGFRα	Cell Signaling (5241)	1:1000	1:50
TTF1	Cell Signaling (8533)	1:1000	1:80
PAX8	Santa Cruz (sc-	1:500	1:80

2.16 Statistical analyses

All statistical analysis was conducted in SPSS version 15(SPSS, Chicago, IL, USA). Descriptive statistics were used to present the study variables. Means ± standard error of mean (SEM) were presented for continuous variables, while frequency and percentages were reported for categorical variables. For all analyses, a p-value <0.05 was considered statistically significant.

For Chapter 3, the correlations between two categorical variables were assessed using Pearson's chi-square tests. For cell frequencies less than 5 Fisher's exact test were reported. For all analyses, frequencies for the strong and mild staining were combined and compared to that of the minimal staining. Correlations of continuous variables were tested using the Spearman's Rho test as the data was non-normally distributed. Classical 2×2 tables were used for estimating the diagnostic performance of markers by calculating specificity, selectivity, positive predictive value (PPV), negative predictive value (NPV) and accuracy. The calculated 95% confidence intervals (CIs) were sufficient to establish the diagnostic superiority of one marker over the other.

For Chapter 5, the correlations between protein expression and metastatic status were assessed using Fisher's exact test for tables and Spearman rank correlation for continuous 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 for analyzing the 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 survival curves.

2.17 References

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3. Tang, X., Benesch, M. G., Dewald, J., Zhao, Y. Y., Patwardhan, N., Santos, W. L., et al (2014). Lipid phosphate phosphatase-1 expression in cancer cells attenuates tumor growth and metastasis in mice. Journal of lipid research, 55(11), 2389-2400.

CHAPTER THREE: THE EXPRESSION OF PLATELET-DERIVED GROWTH FACTOR-AA AND PDGFRα IS PROGNOSTIC OF NODAL METASTASIS IN PAPILLARY THYROID CANCERS.

3.1 Introduction

Approximately 25-40% of PTC cases are managed with surgery and/or RAI ablation therapy to control the disease (1, 2). However, about 20% of those patients harbor metastatic tumors and infiltrations that are resistant to RAI ablation (1, 2). As a result, these patients suffer disproportionate morbidity as they are often subjected to repeated surgical resections, as well as high-doses of the RAI (2). As yet, there are no clinically utilized biomarkers of metastasis in papillary thyroid tumors, and the morphologic predictors of behaviour are inadequate. Nodal metastases or disease recurrence cannot be predicted through histological assessments alone. In fact, about 25% of FNAB-based diagnoses of thyroid malignancy are pronounced indeterminate, and the concerned patients undergo diagnostic hemithyroidectomy. In reality, nearly 80% of these patients do not have cancer. (3-5). When the biopsy results are indeterminate, molecular diagnostics that distinguish benign tumors from malignant ones are commercially available. However, this diagnostic tool often leads to exaggerated treatment of indolent larger tumors, while the aggressive smaller ones with high propensity for nodal spread are missed. In essence, the clinical capacity to successfully predict poor outcomes is lacking (6).

Numerous research endeavours have focused on dysregulated MAPK/Erk pathway as the defining factor in PTC malignancy (7, 8). However, outlining this

pathway has not produced biomarkers with valuable clinical benefits, especially those that will aid metastatic risk prediction. As such, accurate selection of patients whose tumors have high metastatic potentials, and require aggressive treatment regimens like prophylactic level VI lymph node dissection, remains challenging for clinicians. Clearly, there is an urgent unmet need for biomarkers that will aid the stratification of PTCs according to their metastatic potential. A successful search for such markers will require a better understanding of the pathways that drive thyroid tumor growth and metastases. A strong positive association between the expression of PDGFRα and nodal metastasis in papillary thyroid tumors has been documented (9). In this chapter, we expanded this previous finding by assessing the activation status of PDGFRα in the metastatic disease, by exploring the distribution of PDGF ligands in clinical samples. Ultimately, we proposed that the expression of PDGFRα and its associated ligands will be predictive of nodal metastasis in PTC cases.

3.2 Results

Demographics

The clinical and demographic data of the patients have been compiled into **Table 3.1**. The tissue microarrays (TMA) used contained 229 formalin-fixed, paraffin-embedded archived samples including 55 follicular adenomas and 162 papillary thyroid carcinomas (103 with and 59 without nodal metastases) and 12 lymph node metastasis. The mean age for follicular adenoma diagnosis was 51.5 years compared to 48.8 and 45.2 years for node negative and positive primary PTCs respectively. Most of the follicular adenoma (67%), node negative PTC (85%) and node positive PTC (81%) patients, were females. Average tumor sizes (in cm) for follicular adenoma as well as node negative and positive PTC was 3.7, 2.5 and 2.6 respectively.

PDGF ligand/Receptor Expression

PDGF ligands AA, BB, and DD were selected for surveillance by immunohistochemistry. Two other ligands, PDGF-AB and PDGF-CC were not studied here. Instead, we selected two ligands that selectively activate each of the homodimeric forms of the PDGF receptors α and β, as well a third one which interacts with all three receptor combinations including the heterodimeric form. PDGF-BB interacts with all three receptor combinations (αα, ββ, and αβ) with high affinity. PDGF-AA is selective for PDGFRαα while PDGF-DD is regarded as a

PDGFRββ specific agonist, even though it binds PDGFRαβ with a significantly lower affinity (17, 18). Moreover, the relevance of PDGF-AB and PDGF-CC in cancer biology is not well documented.

The immunohistochemical staining results across the histological subtypes are summarized in **Table 3.2** and representative images are shown in **Fig. 3.1.** Staining for each case was evaluated in triplicate without prior knowledge of the clinical outcome. Cytoplasmic staining intensity for all markers was quantified as follows: 0 as minimal, 1 as mild, and 2 as strong. Incomplete or fragmented sample cores on the tissue array were not scored. Majority of the node positive primary PTC (88%) and lymph node metastases (83%) stained mildly or strongly for PDGF-AA. Similarly, all (n=59) of the node positive primary PTC and 92% of the nodal metastases, exhibited mild or strong staining intensity for PDGF-BB. Only 53% of the node positive primary PTC and 67% of the nodal metastases displayed mild or strong staining intensity for PDGF-DD. Out of 103 node negative PTC cases, mild or strong immuno-reactivity with ligands AA, BB, and DD was observed in 39%, 96%, and 44% respectively. Many of the 55 follicular adenoma lesions stained minimally for the PDGF-AA (73%), while only 18% and 51% exhibited the same staining intensity for PDGF-BB and PDGF-DD. PDGFRα was either mildly or strongly expressed by 86% of node positive PTC tissues and 92% of the nodal metastases, while only 29% of the node negative PTCs showed the same staining intensity.

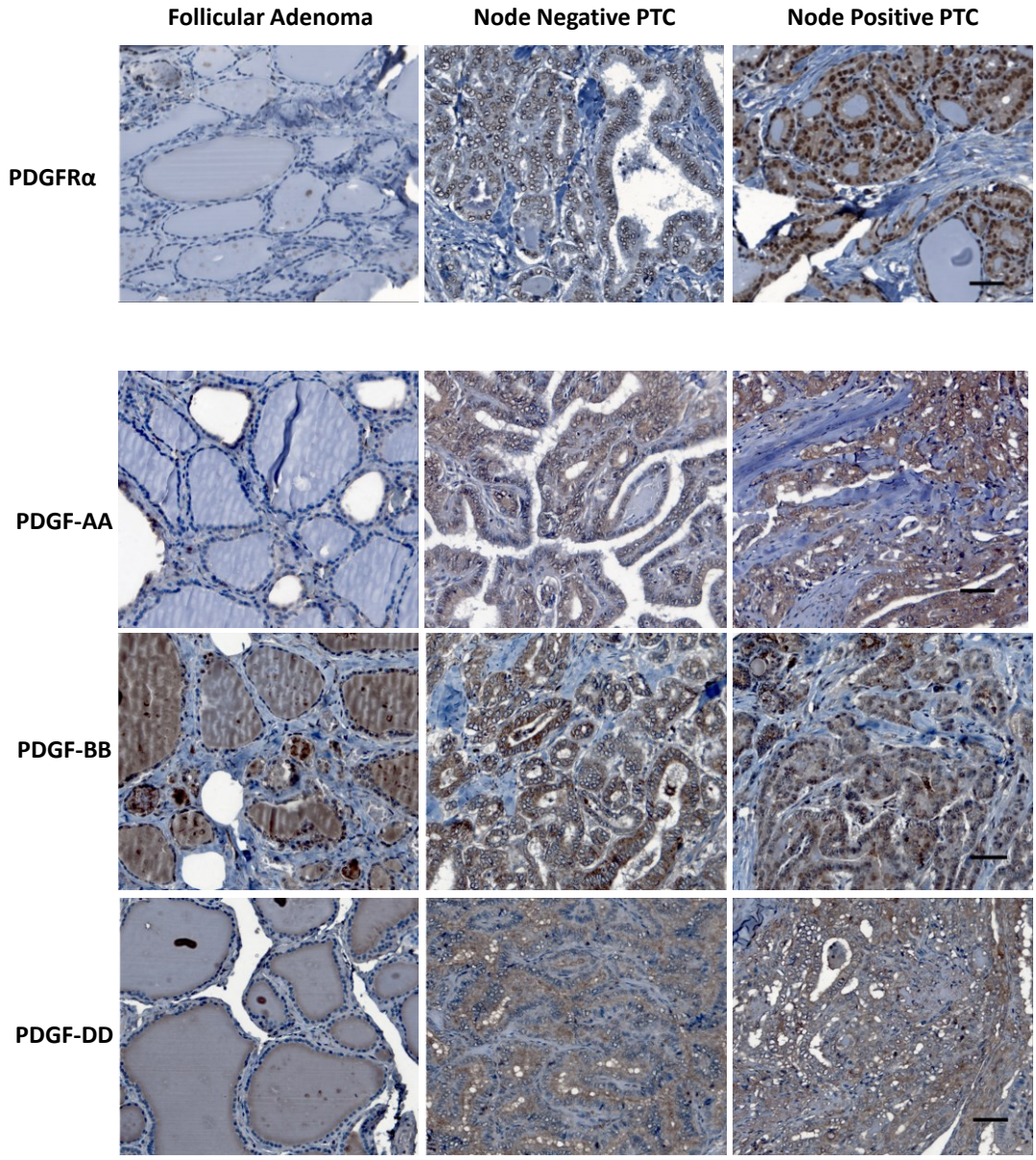

Figure 3.1: Representative immune-histochemical staining of PDGF-AA, PDGF-BB, PDGF-DD and PDGFRα in benign and neoplastic thyroid tissues. Scale bar = 50 μM

Table 3.1 Clinical and demographic characteristics of 229 patients

	n	Age	Sex	Tumor size (cm)
Histological Subtypes		Mean±SEM	Female (%)	Mean±SEM
Follicular Adenoma	55	51.5 ± 14.5	67	3.7 ± 2.0
Node negative PTC	103	48.8 ± 16.1	85	2.5 ± 1.6
Node positive PTC	59	45.2 ± 14.8	81	2.6 ± 1.3
Lymph Node Metastases	12	47.3 ± 12.8	75	2.3 ± 2.5

Table 3.2 Immunoreactivity for PDGFRα, PDGF AA, PDGF BB, and PDGF DD in 229 benign lesions and tumors from primary PTCs with and without nodal metastasis

Markers	**Histological Subtype**	**Percentage of cases (%)**	
		Staining Intensity	
		0	**1 and 2**
PDGFRα	Follicular Adenoma	76	24
	Node negative PTC	71	29
	Node positive PTC	14	86
	Lymph Node Metastases	8	92
PDGF-AA	Follicular Adenoma	73	27
	Node negative PTC	61	39
	Node positive PTC	12	88
	Lymph Node Metastases	17	83
PDGF-BB	Follicular Adenoma	18	82
	Node negative PTC	4	96
	Node positive PTC	0	100
	Lymph Node Metastases	8	92
PDGF-DD	Follicular Adenoma	51	49
	Node negative PTC	56	44
	Node positive PTC	47	53
	Lymph Node Metastases	33	67

Similar to PDGF-AA staining, PDGFRα staining was minimal or not detected in majority (76%) of the follicular adenomas. Using cell sorting techniques, we also reveal that primary thyroid carcinomas lacking metastases exhibited low levels of PDGFRα, while metastatic PTC specimens revealed much higher levels of PDGFRα on the cell surface (P<0.0001) (**Fig. 3.2**).

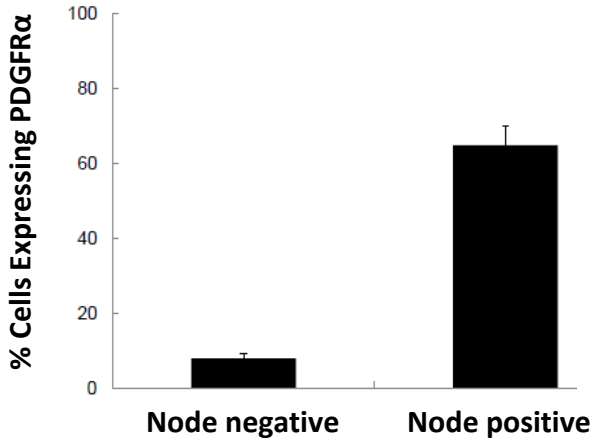

Figure 3.2: PDGFRα expression in fresh tumor isolates. Quantitative analysis using flow cytometry of freshly isolated primary tumors comparing PDGFRA positive cells in primary tumors lacking metastases (n=4) and those with proven metastatic disease (n=6), p<0.0001.

PDGF-AA is associated with metastasis and PDGFRα expression in PTCs

Comparing node negative and positive PTC tumors, we further determined if the staining intensity of ligands was associated with nodal status. Of all 3 ligands under assessment, only PDGF-AA expression was significantly associated with the nodal status (p = 0.0001, **Table 3.3**). PDGF-AA positive PTC cases were more likely to have nodal metastasis compared to the PDGF AA negative cases. No statistically relevant relationship seemed to exist between PTC nodal status and the staining intensity of PDGF-BB (p = 0.298) and DD (p = 0.327).

PDGFRα is known for its strong positive association with nodal metastasis in PTCs (26), and the data in **Table 3.2** further supports this relationship. Given that majority (88%) of node positive PTC samples stained mildly or strongly for PDGF-AA, we hypothesized that a strong association between PDGF-AA and PDGFRα expression exists. In all 162 PTC cases examined, logistic regression analysis (**Table 3.4**) showed that the PDGF-AA expression was significantly associated with the presence of PDGFRα (Odd ratio, OR = 4.6, p = 0.004). The odds of PDGFR staining to be mild and strong are 4.6 times higher when AA staining is mild or strong. A relationship (Odd ratio, OR = 2.4, p = 0.030) also existed between PDGF-BB and PDGFRα expression, while there was no statistically significant relationship (Odd ratio, OR = 1.2, p = 0.463) between PDGF-DD and PDGFRα expression.

Next, we examined the prognostic value of PDGF-AA and PDGFRα expression to the presence of metastases in PTC, comparing node negative (n=103)

and node positive PTC (n=51) samples. For PDGFRα, the sensitivity was 86% and the specificity was 71%, and for PDGF-AA, it was 88% and 61% respectively (**Table 3.5**).

Table 3.3: PDGF ligands and nodal metastasis in PTCs

Markers	**n**	**Nodal Status (n)**		
		Negative	**Positive**	**p-value**
PDGF-AA				
Minimal	**70**	63	7	**< 0.0001***
Mild and Strong	**92**	40	52	
	162			
PDGF-BB				
Minimal	**4**	4	0	0.298
Mild and Strong	**158**	99	59	
	162			
PDGF-DD				
Minimal	**86**	58	28	0.327
Mild and Strong	**76**	45	31	
	162			

PDGF, platelet-derived growth factor

Table 3.4: Logistic regression model of the association between PDGF ligands and PDGFRα expression

	Univariate analysis	
Variables	**OR (95% CI)**	**p-value**
PDGF-AA	4.6 (1.5–7.3)	**0.004***
PDGF-BB	2.4 (1.0–4.7)	**0.030***
PDGF-DD	1.2 (0.63–2.3)	0.463

PDGF, platelet-derived growth factor; CI, confidence interval; OR, odds ratio

Table 3.5: Diagnostic value of PDGFRα and PDGF-AA for predicting nodal metastatic risk in PTC

Markers	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
PDGFRα	0.86 (0.78 - 0.92)	0.71 (0.61 - 0.80)	0.75 (0.66 - 0.82)	0.84 (0.74 - 0.91)
PDGF-AA	0.88 (0.80 - 0.94)	0.61 (0.51 - 0.71)	0.69 (0.60 - 0.77)	0.84 (0.73 - 0.91)

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor

3.3 Discussion

Lymph node infiltrations are common in patients with papillary thyroid tumor (10, 11), and their occurrence has unfavorable prognostic implications. They are associated with increased risk of locoregional recurrence, and in older patients a greater risk of death from thyroid cancer (11, 12). The sensitivity of preoperative ultrasound for central neck lymph node metastases is reported as low as 10% (13). In this chapter we extend the work of Zhang *et al.* 2012 (9) demonstrating that the signalling axis PDGF-AA/ PDGFRα receptor may be a key component driving nodal metastases in PTC.

First, as judged by the type of PDGF ligands expressed, the activation status of PDGFRα in the metastatic disease was determined. When PDGFRα shared a similar pattern of expression with ligands that selectively activate it, we considered this an indicator of its activation. We observed the distribution of PDGF -AA, -BB and -DD in a cohort of follicular adenomas, primary papillary thyroid tumors and lymph node metastases by immunohistochemistry. Of all the ligands evaluated, only PDGF-AA showed a positive association with nodal metastasis in the PTC cases. The augmented expression of PDGFRα and its selective activator (PDGF-AA) in the metastatic samples is a strong indication that PDGFRα signaling is involved in the nodal spread of papillary thyroid tumours. Even though immunohistochemical positivity is not a complete proof of a functional role, this pattern of expression shows that PDGFRα is more likely to have a functional role in nodal metastasis, than in early-stage PTC development. To support this concept, functional studies to

determine the role of PDGFRα in driving aggressiveness and metastases in PTC are required. Interestingly, not only did the majority of node positive primary PTCs and their metastatic deposits stain mildly or strongly for PDGF-AA and PDGFRα compared to the node negative tumors, we also found a significant association between the expression of this ligand and PDGFRα. Although, the universal ligand, PBGF-BB, had no significant association with PTC metastasis, a mild association with PDGFRα was observed. There was no relationship between PDGFRα expression or metastasis and PDGF-DD staining.

This is the first study demonstrating an up-regulation of both PDGFRα and PDGF-AA, indicating a likely connection between signaling by this pathway and malignancy, possibly metastases. This connection may be partly tied to the well-known role of the PDGF/PDGFR axis in lymphangiogenesis (14-18). Lymphangiogenesis, which refers to the development of new lymphatic vessels from pre-existing ones, is thought to favor the entry of tumor cells into the lymphatic vasculature and potentially promote tumor dissemination to distant sites (19). The extreme vascularity and high propensity for nodal metastasis commonly observed in papillary thyroid tumors highlight the likelihood that they are targets of lymphangiogenic processes. Vascular endothelial growth factor receptor 3 (VEGFR3) is another receptor tyrosine kinase, which drives nodal metastasis in response to the lymphangiogenic growth factors, VEGF-C and VEGF-D. VEGF-C levels in primary tumors have been reported to correlate with lymph node metastasis in lung, gastric, breast, oesophageal, pancreas, prostate, and colorectal cancer (20, 21). Although

increases in serum levels of VEGF-D and -A have been reported in thyroid cancer compared to benign lesions or normal thyroid tissue, no study has verified that the increase in these signaling moieties translates into downstream effector pathways (22).

Attempts at using BRAF gene testing to predict malignancy have been debated, they are not clear markers for metastatic disease despite larger studies indicating higher rates of positivity in malignant disease (23). The idea that BRAF V600E mutation could occur *de novo* as a secondary genetic event driving disease progression, was first conceived when BRAF V600E mutation was detected in nodal deposits from primary PTC tumors that did not harbor the mutation (24). However, on the other hand, the tumorigenicity of BRAFV600E has been well demonstrated in cell line, xenograft models, and transgenic mouse systems (25). Together, these findings suggest that as a single genetic event, BRAF V600E, is able to initiate PTC oncogenesis and progression. Interestingly, some authors have found intra-tumor heterogeneity in the distribution of BRAF V600E mutation within human PTC tumors - majority of tumor cells harbored the wild-type BRAF, while less than 50% harbored the BRAF V600E mutation (26). This incidence of BRAFV600E in only a subpopulation of PTC cells also presents this genetic alteration as a late secondary genetic event in PTC. However, another plausible explanation is that once PTC is initiated by BRAF V600E, other secondary oncogenic alterations take over to drive disease progression, including nodal metastasis, such that BRAF mutation is no longer selected for (25).

The reality is in some geographic catchments, the positivity rate of BRAF mutations is in excess of 60% and this makes for a poor diagnostic test when the pre-existing prevalence is so high in the population. In fact, a recent report showed that even though BRAF V600E mutations are more prevalent than previously thought in pediatric PTC patients, they do not correlate with aggressive disease features (27). Further evidence of this controversy was exposed, when a group of authors who initially found a positive correlation between BRAF V600E mutation and macroscopic lymph nodes metastases (28), did not find any correlation with micrometastases (29). Here, we demonstrated that the PDGF-AA levels are relatively low in the absence of nodal metastasis, and when combined with PDGFRα staining, may provide a useful adjunct with more sensitivity and specificity.

Finally, we evaluated the performance of PDGF-AA and PDGFRα in identifying aggressive variants of PTC with high metastatic potentials. Both PDGF-AA and PDGFRα showed relatively high sensitivity, but lower specificity. Overall, we have demonstrated that PDGF-AA and PDGFRα expression are majorly augmented in PTC metastasis, suggesting that they could be valuable prognostic markers of metastatic risk. This ability to differentiate indolent PTC from that with high metastatic potentials could prevent future recurrences or distant metastasis by allowing for more aggressive treatment. This will help oncologists to effectively direct aggressive management to the patients who need it, while also, saving patients with indolent disease from unnecessary (and costly) treatment. With the increased awareness that lymphangiogenesis is an important step in cancer metastasis (30), the

blockade of PDGFRα signaling also presents as an attractive therapeutic strategy against metastatic spread in situations of high PDGF-AA and PDGFRα expression.

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CHAPTER FOUR: PDGFRα INDUCES A MIGRATORY PHENOTYPE WITH INVADOPODIA PROTRUSIONS THROUGH EPITHELIAL MESENCHYMAL TRANSITION IN PTC CELLS.

4.1 Introduction

As shown in Chapter 3, it appears that PDGFRα signaling contributes to the acquisition of metastatic phenotypes in PTC. The elevated expression of PDGF-AA (a selective activator of PDGFRα) in metastasis patient samples, support this notion. Certainly, the signaling pathways and phenotypic adaptations harnessed by PDGFRα to confer pro-metastatic transformations in PTC cells require more understanding. In quaternary and tertiary care settings, tyrosine kinase inhibitors are considered a major part of the therapeutic arsenal against metastatic PTCs, particularly those refractory to RAI treatment. Unfortunately, the varied outcomes and adverse side effects which accompany their empirical use underscores the need for a much better understanding of the cellular processes driving metastatic disease in PTC (1).

Here, we explored the phenotypic alterations driven by PDGFRα activation in human PTC cells and the downstream signaling cascades through which they are effected. Particularly, the migratory behavior and three dimensional architecture of PTC cells were examined in constructs lacking or overexpressing PDGFRα. Furthermore, we surveyed the contributions of the downstream signaling pathways, PI3K/Akt, MAPK/Erk, STAT3 and Wnt/β-catenin, to the PDGFRα-induced behavioral changes. As a therapeutic strategy, targeted inhibition of PDGFRα activity

in PTC cells was also examined, given the success of this approach in other cancers (2).

4.2 Results

Inducible Expression of PDGFRα in PTC Cells and Specificity of Receptor Activation

PTC cells lacking or expressing PDGFRα on the same genetic background were compared to understand how PDGFRα drives aggressiveness. Although BCPAP cells express PDGFRβ receptor as expected (3), they lack significant expression of PDGFRα (**Fig. 4.1A)**. These cells were stably transduced with lentiviral vectors to express PDGFRα (BCPAP-PDGFRα) under doxycycline induction (**Fig. 4.1B**). Control cells (BCPAP-Empty) contained only the empty vector.

The PDGF ligands bind to and activate PDGFRα and PDGFRβ with different specificities (4-6). Ligand binding causes receptor dimerization, a key event in activation, which brings the intracellular domains of the receptors together to promote autophosphorylation. The specificity of receptor activation in response to the ligand isoforms AA, BB and DD was confirmed in PTC cells. As noted in other cell types (4-6), PDGF-BB activates PDGFRα and PDGFRβ, whereas PDGF-AA preferentially activates PDGFRα with no effect on PDGFRβ (**Fig. 4.1C**). Ligands BB and DD induced PDGFRβ phosphorylation whether it was expressed alone (BCPAP-Empty) or with PDGFRα (BCPAP-PDGFRα). PDGF-BB exhibited the same pattern of interaction with PDGFRα, whereas PDGF-DD did not activate PDGFRα when it was expressed alone as seen in 8305C, another thyroid cancer cell line (**Figs. 4.1A and 4.1D**). Taken together, these results further demonstrate that PDGF-AA and -BB activate PDGFRαα receptor homodimers, whereas DD and BB will activate ββ

receptor homodimers as well as the αβ receptor heterodimers in PTC cells. In addition, 50 ng/ml of the ligands was sufficient to produce considerable levels of receptor phosphorylation (**Fig. 4.1C**). Time-dependent activation of PDGFRα in response to 50 ng/ml PDGF-AA is also shown in **Fig. 4.1E**. As indicated by the phosphorylation of residues Tyr754 and Tyr849, PDGFRα activation peaked between 6 to 30 min of PDGF-AA treatment but disappeared after 8 h of exposure.

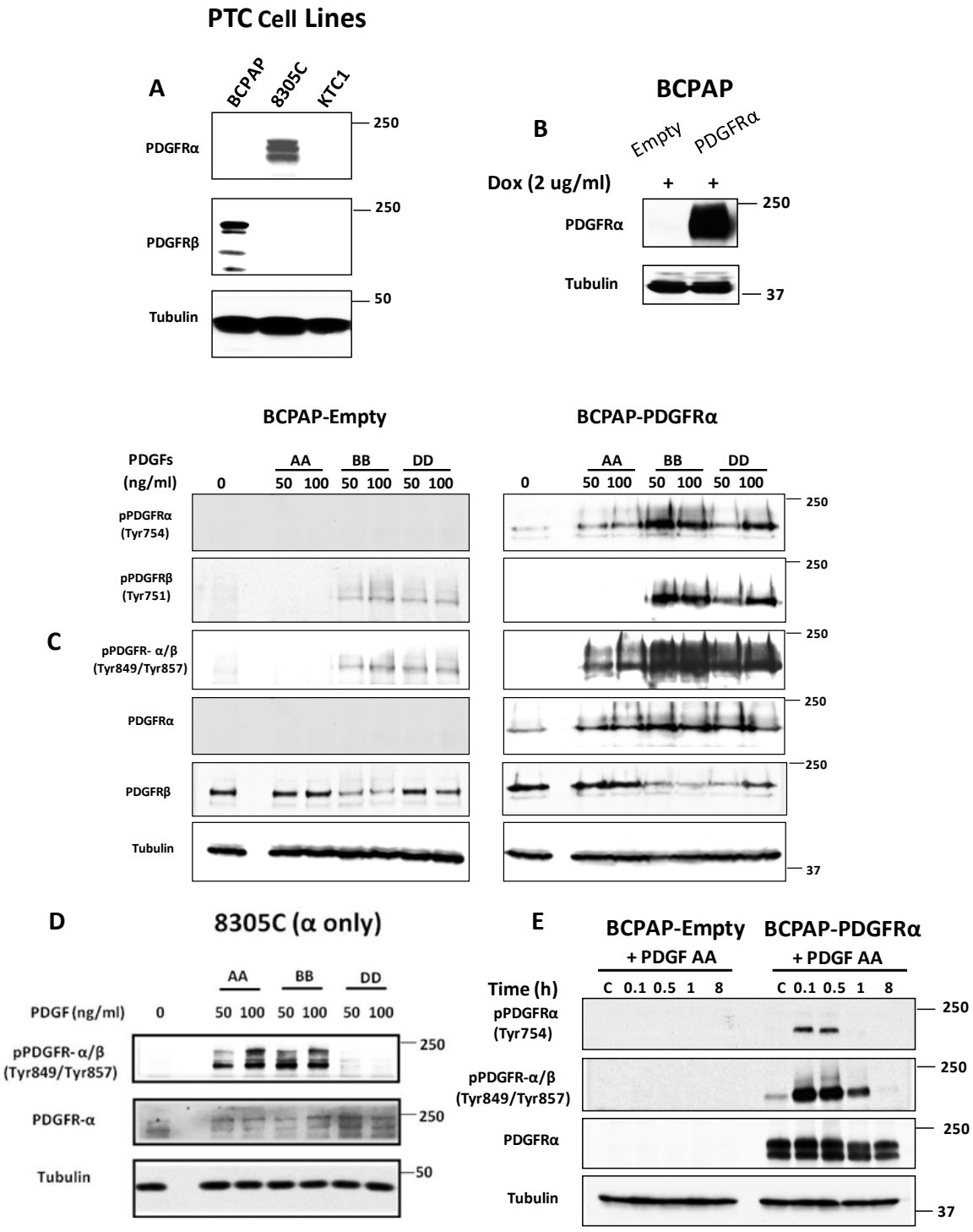

PTC Cell Lines
A
BCPAP
8305C
KTC1
PDGFRα
PDGFRβ
Tubulin
250
250
50
B
BCPAP
Empty
PDGFRα
Dox (2 ug/ml)
+ +
PDGFRα
Tubulin
250
37
C
BCPAP-Empty
BCPAP-PDGFRα
PDGFs
(ng/ml)
0
AA
BB
DD
50 100 50 100 50 100
pPDGFRα (Tyr754)
pPDGFRβ (Tyr751)
pPDGFR- α/β (Tyr849/Tyr857)
PDGFRα
PDGFRβ
Tubulin
250
37
D
8305C (α only)
PDGF (ng/ml)
pPDGFR- α/β (Tyr849/Tyr857)
PDGFR-α
Tubulin
50
E
BCPAP-Empty
BCPAP-PDGFRα
+ PDGF AA
Time (h)
C 0.1 0.5 1 8
pPDGFRα (Tyr754)
pPDGFR-α/β (Tyr849/Tyr857)
PDGFRα
Tubulin

Figure 4.1: Inducible expression of PDGFRα in PTC cells and specificity of receptor activation. (A) Western blot analysis showing the native expression profile of PDGFR -α and β in PTC cell lines BCPAP, 8305C and KTC1. (B) Doxycycline-inducible expression of PDGFRα in BCPAP cells (BCPAP-PDGFRα). Control cells containing only the empty vector (BCPAP-Empty) were also generated. PDGFRα protein expression was confirmed in cells after 48 h treatment with 2 μg/ml doxycycline (Dox). (C) BCPAP-Empty cells (native PDGFRβ only) and BCPAP-PDGFRα cells (with both PDGFR –α and β), were serum-starved for 24 h, stimulated for 6 min with the PDGF ligands AA, BB and DD, then phosphorylation was detected for PDGFRα and PDGFRβ. (D) 8305C cells (native PDGFRα) was stimulated for 6 min with the PDGF ligands AA, BB and DD and probed for PDGFRα phosphorylation. (E) Serum-starved BCPAP-Empty and BCPAP-PDGFRα cells were treated or untreated with 50 ng/ml PDGF-AA and cell lysates were collected at the indicated times. The time course for PDGFRα phosphorylation on Tyr754 and Tyr849 was analyzed by western blotting. Tubulin was used as the loading control. The results are representative of 4 independent experiments.

PDGFRα Confers a Branched 3D Morphology and Migratory Phenotype

Given the specificity of PDGF-AA for the PDGFRα subunit, we further unraveled the phenotypic consequences of preferentially activating PDGFRα with this ligand. Previous studies suggest strongly that the morphology of cells is more accurately recreated *in vitro* by using 3D culture systems (7, 8). Matrigel culture closely mimics physiological conditions (9, 10) and so we investigated the effect of PDGFRα expression and activation on cell morphology by comparing BCPAP-Empty and BCPAP-PDGFRα cells propagated in growth factor reduced Matrigel. **Fig. 4.2A** confirmed the over-expression of PDGFRα mRNA in BCPAP-PDGFRα cells relative to BCPAP-Empty cells after growing in 3D Matrigel matrix for 7 days. BCPAP cells expressing PDGFRα exhibited a highly branched 3D architecture with membrane protrusions (52 % ± 5.5 of structures were branched) (**Figs. 4.2B and 4.2C**). Treatment with 50 ng/ml PDGF-AA potentiated the formation of the branched structures by ~2-fold (94± 2%). In contrast, control cells lacking PDGFRα formed dense and compact spheroids with no branching in the absence or presence of PDGF-AA. In agreement with this, 8305C cells, which natively express PDGFRα showed branched 3D architecture, whereas branching was undetectable in KTC1 cells, which lack the receptor (**Fig. 4.3A**). Then, we assessed primary cultures from patient tumors, and found that the PDGFRα-induced 3D growth pattern was consistent. Primary thyroid follicular cells lacking PDGFRα grew into thyrospheres, while those with native PDGFRα expression developed irregular membrane protrusions (**Fig. 4.3B**).

Aggressive cancer cells acquire the ability to migrate to secondary sites to establish metastases and so we investigated the consequence of PDGFRα activation on PTC cell migration. PDGF-AA stimulated the migration of BCPAP-PDGFRα cells by about 3-times more than the control BCPAP-Empty cells (**Fig. 4.2D**). There was no significant difference in basal migration between both cell groups in the absence of PDGF-AA. The expression of PDGFRα in BCPAP cells had no significant effect on cell proliferation ($p=0.08$) in agreement with a previous report in breast cancer cells (11). This eliminates the possibility that the observed changes in migration over 4 h resulted from any increase in cell division.

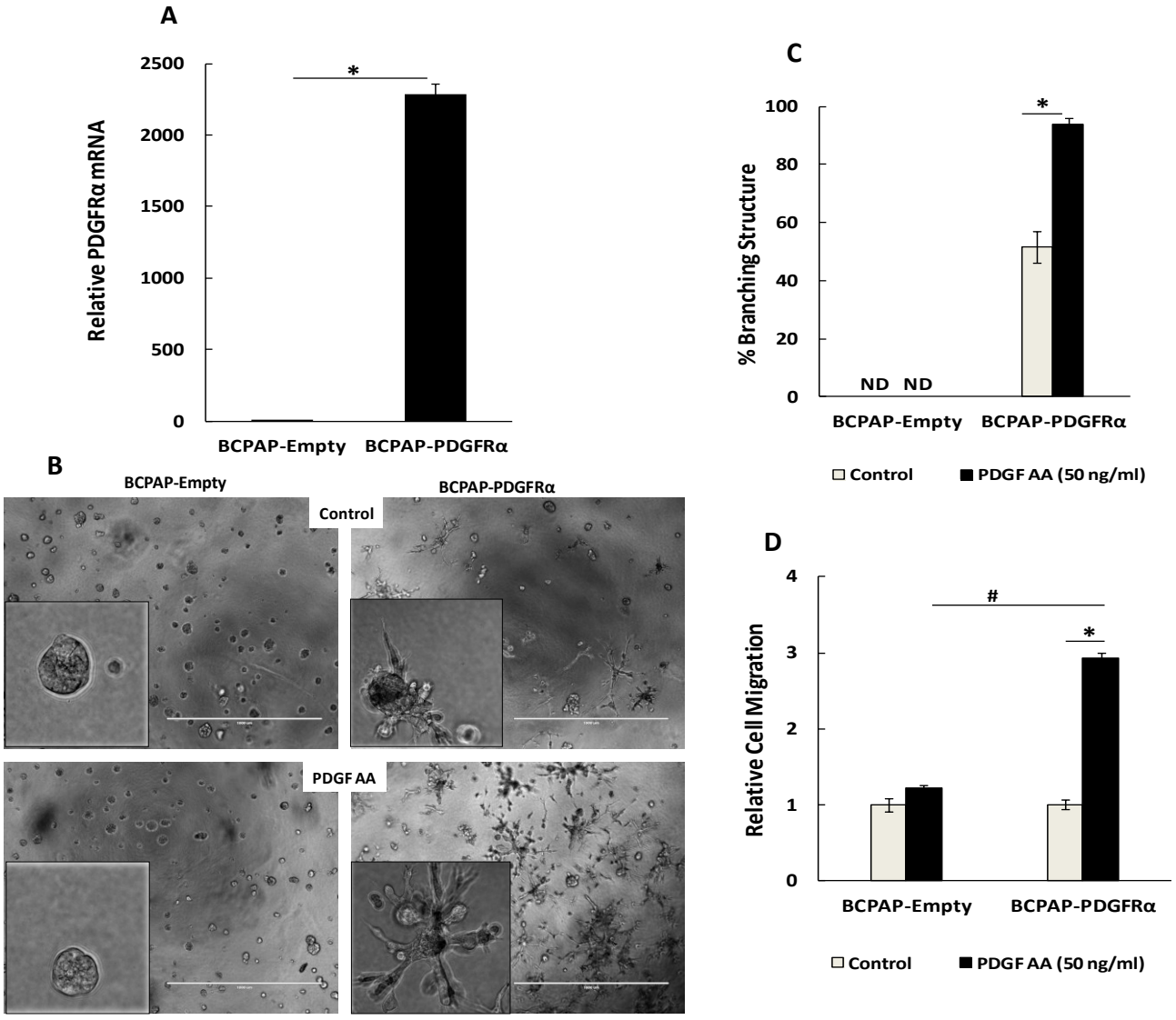

A
Relative PDGFRα mRNA
2500
2000
1500
1000
500
0
*
BCPAP-Empty
BCPAP-PDGFRα
B
BCPAP-Empty
BCPAP-PDGFRα
Control
PDGF AA
C
% Branching Structure
100
80
60
40
20
0
*
ND ND
BCPAP-Empty
BCPAP-PDGFRα
Control
PDGF AA (50 ng/ml)
D
Relative Cell Migration
4
3
2
1
0
#
*
BCPAP-Empty
BCPAP-PDGFRα
Control
PDGF AA (50 ng/ml)

Figure 4.2: PDGFRα expression and activation conferred an invasive-like, branching 3D morphology and a migratory phenotype on papillary thyroid cancer cells. (A) Relative PDGFRα mRNA expression in BCPAP-Empty and BCPAP-PDGFRα cells after 7 days of 3D culture. **(B-C)** BCPAP-Empty and BCPAP-PDGFRα cells were cultured in Matrigel for 7 days in the absence (control) or presence of 50 ng/ml PDGF-AA. **(B)** Representative phase contrast images (bar = 1000 μm) show dramatic induction of a branching morphology upon PDGFRα expression (upper right) and activation with PDGF-AA (lower right). The left panels show the contrasting 3D morphology of the control BCPAP-Empty cells. **(C)** The number of branching structures was presented as a percentage of the total number of structures observed. ND denotes branching structures not detected. **(D)** PDGF-AA-induced migration of BCPAP-PDGFRα cells was about 3 times greater compared to BCPAP-Empty cells. For relative quantitation, the value of 1 was arbitrarily assigned to BCPAP-Empty cells. Results in **C and D** are means ± SEM, n = 6. Significant differences are indicated by *, $p < 0.05$ compared with untreated control cells, and #, $p < 0.05$ compared with corresponding samples of control BCPAP-Empty cells.

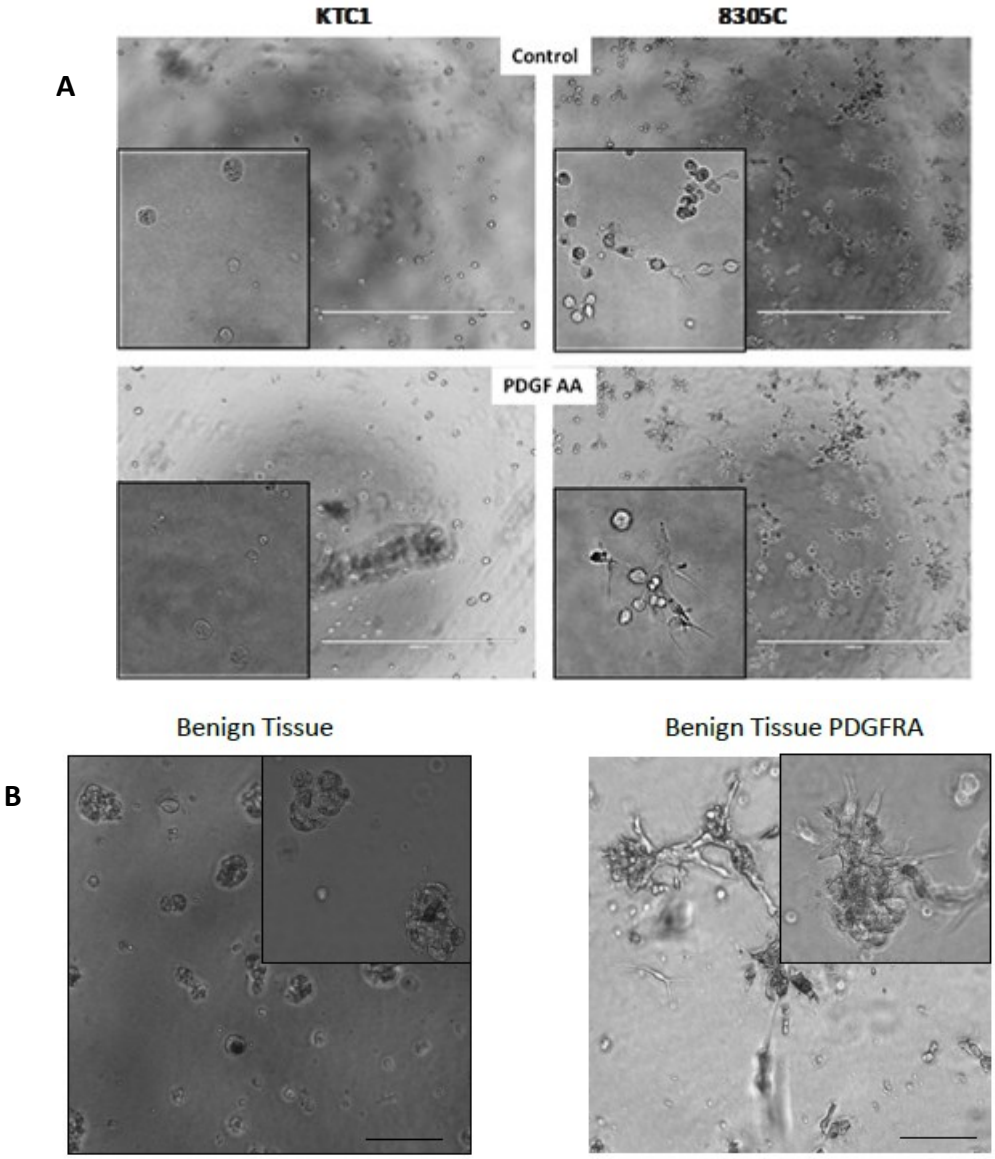


Figure 4.3: PDGFRα promotes branching in 3D culture (A) 8305C cells (with native PDGFRα expression) and KTC1 cells (without PDGFRα expression) were grown in 3D Matrigel culture for 7 days. While 8305C cells exhibited branched 3D morphology, KTC1 cells produced dense spheroids in the absence and presence of PDGF-AA. Scale bars = 1000 μm **(B)** 3D culture assessment of varying growth patterns between PDGFRα-positive and negative thyroid primary cultures. Scale bars 200 μm.

PDGFRα Signaling and Its Downstream Effector Pathways in PTC Cells

The downstream signal transduction cascades provoked by PDGFRα activation were investigated to further understand the striking phenotypic changes described above. PDGFRα stimulates multiple downstream signaling pathways in cancer cells (12); but this study focused on the MAPK/Erk, PI3K/Akt, STAT3, and the Wnt/βcatenin pathways due to their well-documented association with thyroid cancer development and progression (13-16). Pathway activation was assessed in BCPAP-PDGFRα cells after the homodimeric form of PDGFRα was stimulated with PDGF-AA (given the results in **Fig. 4.1C**) and specifically blocked with the TKI, Crenolanib (17, 18). After pretreatment with various concentrations of Crenolanib (0.1, 0.5, and 1 μM) for 1 h, cells were exposed to PDGF-AA for 6 min. The phosphorylation levels of Erk, Akt, STAT3 and GSK3β served as indicators for Erk/MAPK, PI3K/Akt, STAT3, and the Wnt/βcatenin pathway activation, respectively. Upon Wnt pathway activation, GSK3β is inactivated by its phosphorylation on Ser9, allowing for cytoplasmic accumulation of β-catenin which is the primary effector of the Wnt/βcatenin pathway. β-catenin promotes the transcription of Wnt-target genes (16).

Increased PDGFRα phosphorylation on residues Tyr754 and Tyr849, which corresponds with significant upregulation of Akt phosphorylation was observed with PDGF-AA treatment (**Fig. 4.4A**). When cells were pretreated with Crenolanib, PDGFRα phosphorylation was suppressed to comparable levels while Akt phosphorylation was completely blocked with all Crenolanib concentrations (**Fig.**

4.4A). The induction of Erk, STAT3 (Tyr705) and GSK3 (Ser9) phosphorylation after this short exposure to PDGF-AA was very weak. However, Crenolanib significantly inhibited their phosphorylations in a dose-dependent manner. When BCPAP-PDGFRα cells were exposed to increasing concentrations of PDGF-AA for 15 min, phosphorylated Akt and GSK3β increased with increasing doses of PDGF-AA (**Fig. 4.4B**). By contrast, the PDGF-AA-stimulated increase in phosphorylated Erk and STAT3 was weak and independent of dose.

The sensitivity of BCPAP-Empty and BCPAP-PDGFRα to the indicated concentrations of Crenolanib is documented in **Fig. 4.4C**. Both cell types responded similarly to Crenolanib treatment. While 1 μM Crenolanib, blocked cell growth by approximately 40%, no significant inhibition was observed with 0.1 μM and 0.5 μM.

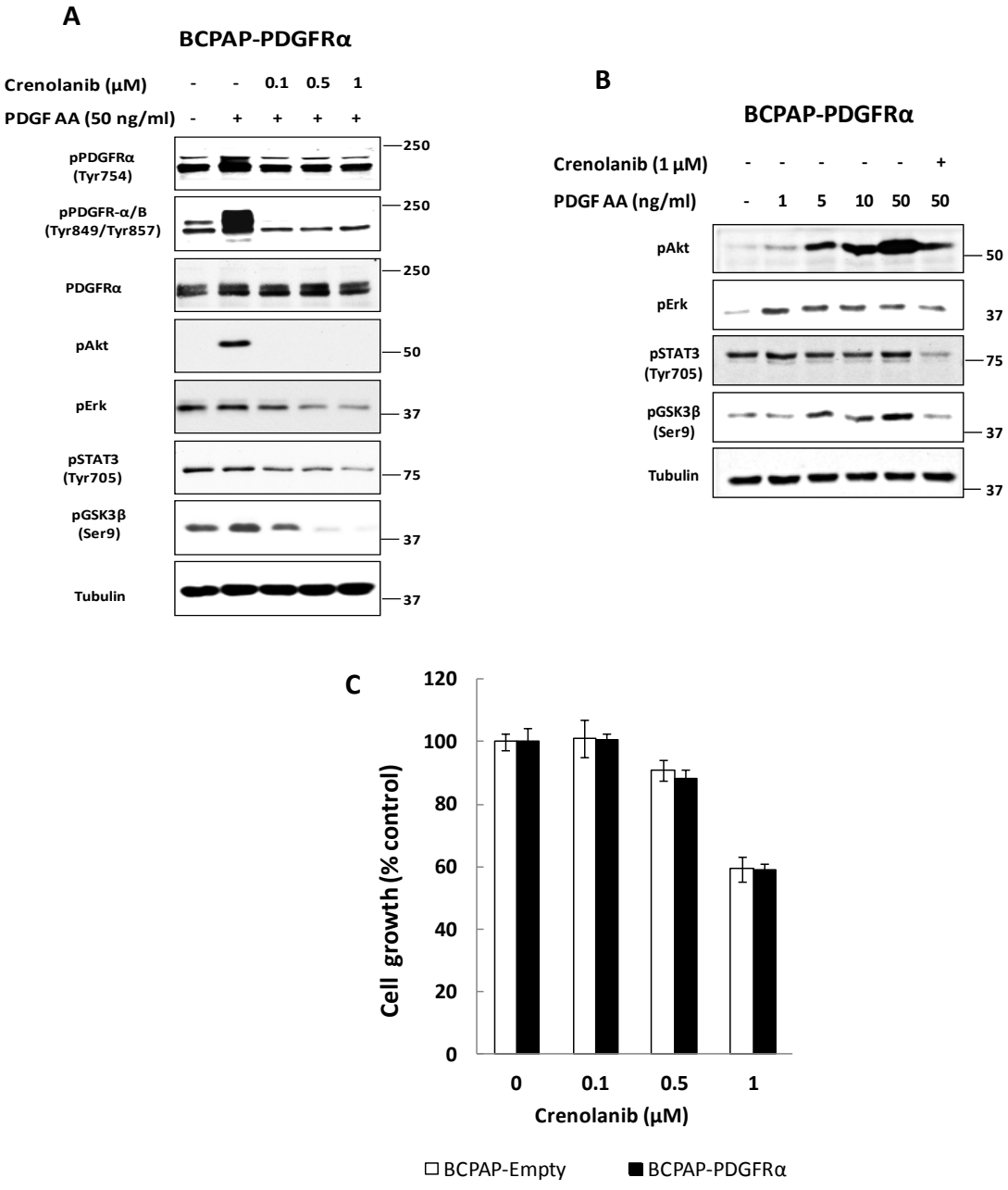

A
BCPAP-PDGFRα
Crenolanib (μM) - - 0.1 0.5 1
PDGF AA (50 ng/ml) - + + + +
pPDGFRα (Tyr754)
pPDGFR-α/B (Tyr849/Tyr857)
PDGFRα
pAkt
pErk
pSTAT3 (Tyr705)
pGSK3β (Ser9)
Tubulin
250
250
250
50
37
75
37
37
B
BCPAP-PDGFRα
Crenolanib (1 μM) - - - - - +
PDGF AA (ng/ml) - 1 5 10 50 50
pAkt
pErk
pSTAT3 (Tyr705)
pGSK3β (Ser9)
Tubulin
50
37
75
37
37
C
Cell growth (% control)
120
100
80
60
40
20
0
0 0.1 0.5 1
Crenolanib (μM)
BCPAP-Empty
BCPAP-PDGFRα

Figure 4.4: PDGFRα signaling and its downstream effector pathways in PTC cells. (A) Pharmacological inhibition of PDGFRα activity with Crenolanib was confirmed by western blot. BCPAP-PDGFRα cells were serum-starved for 24 h, pretreated with or without the indicated Crenolanib doses for 1 h, followed by 6 min stimulation with 50 ng/ml PDGF AA. Cells unexposed to both Crenolanib and PDGF-AA served as control. Cell lysates were subjected to western blot analyses and equal protein loading was confirmed with tubulin expression. **(B)** Serum-starved BCPAP-PDGFRα cells were pretreated with or without 1 μM Crenolanib for 1 h and stimulated with or without the indicated concentrations of PDGF-AA for 15 min followed by western blot analyses of Erk, Akt, STAT3 and GSK3β phosphorylation. Tubulin was used as the loading control. The results are representative of 3 independent experiments. **(C)** The effect of Crenolanib on the growth of BCPAP-Empty and BCPAP-PDGFRα cells following a 5-day treatment with the indicated concentrations as measured by MTS assay. Cell growth inhibition was expressed as a percentage of the untreated control cells.

STAT3 Signaling Plays an Important Role in the PDGFRα-associated phenotypes

The role of the Erk/MAPK, PI3K/Akt, STAT3, and Wnt/βcatenin pathways in establishing the PDGFRα-associated phenotypes were studied by inhibition with U0126, LY294002, Stattic and Quercetin, respectively. In cells expressing PDGFRα, PDGF-AA-induced phosphorylation of Akt, Erk, and STAT3 was completely abrogated by LY294002, U0126, and Stattic, respectively (**Fig. 4.5A**). Stattic also had a considerable inhibitory effect on PDGF-AA-mediated Akt phosphorylation. This is compatible with a recent report in which Stattic inhibited Akt phosphorylation in cardiac cells (19). Quercetin, a potent inhibitor of β-catenin transcriptional activity (20) significantly reduced ligand-activated Akt phosphorylation levels. Surprisingly, treatments with LY294002, Stattic, and Quercetin strongly induced Erk phosphorylation in a PDGF-independent manner. This finding is consistent with significant Erk activation following exposure to LY294002 in lymphoma cells (21). Activation of the MAPK/Erk pathway may be a compensatory response to the blockade of the Akt, STAT3 and Wnt/β-catenin pathways.

While the inhibition of PI3K/Akt, and STAT3 pathways reduced PDGF-AA-stimulated migration of PDGFRα expressing cells, blockade of MAPK/Erk and Wnt/βcatenin had no significant effect (**Fig. 4.5B**). STAT3 blockade produced the most significant anti-migratory effect such that the migratory ability of Stattic-treated cells was comparable with that of untreated control cells. Blockade of the MAPK/Erk, PI3K/Akt and STAT3 pathways in PDGFRα expressing cells significantly reverted

the formation of branched 3D structures in culture, while the effect of Wnt/βcatenin pathway inhibition was inconsequential (**Fig. 4.5C**). It is noteworthy that the inhibitor concentrations used in the 3D culture and cell migration experiments had no significant effect on cell viability (**Fig. 4.6**). These MAPK/Erk, PI3K/Akt, STAT3, and Wnt/βcatenin pathways were inhibited with U0126 (10 μM), Ly294002 (10 μM), Stattic (2.5 μM) and Quercetin (10 μM), respectively. In addition, the viability of the PDGFRα-null and PDGFRα-positive BCPAP cells was comparable with each of the varied concentrations.

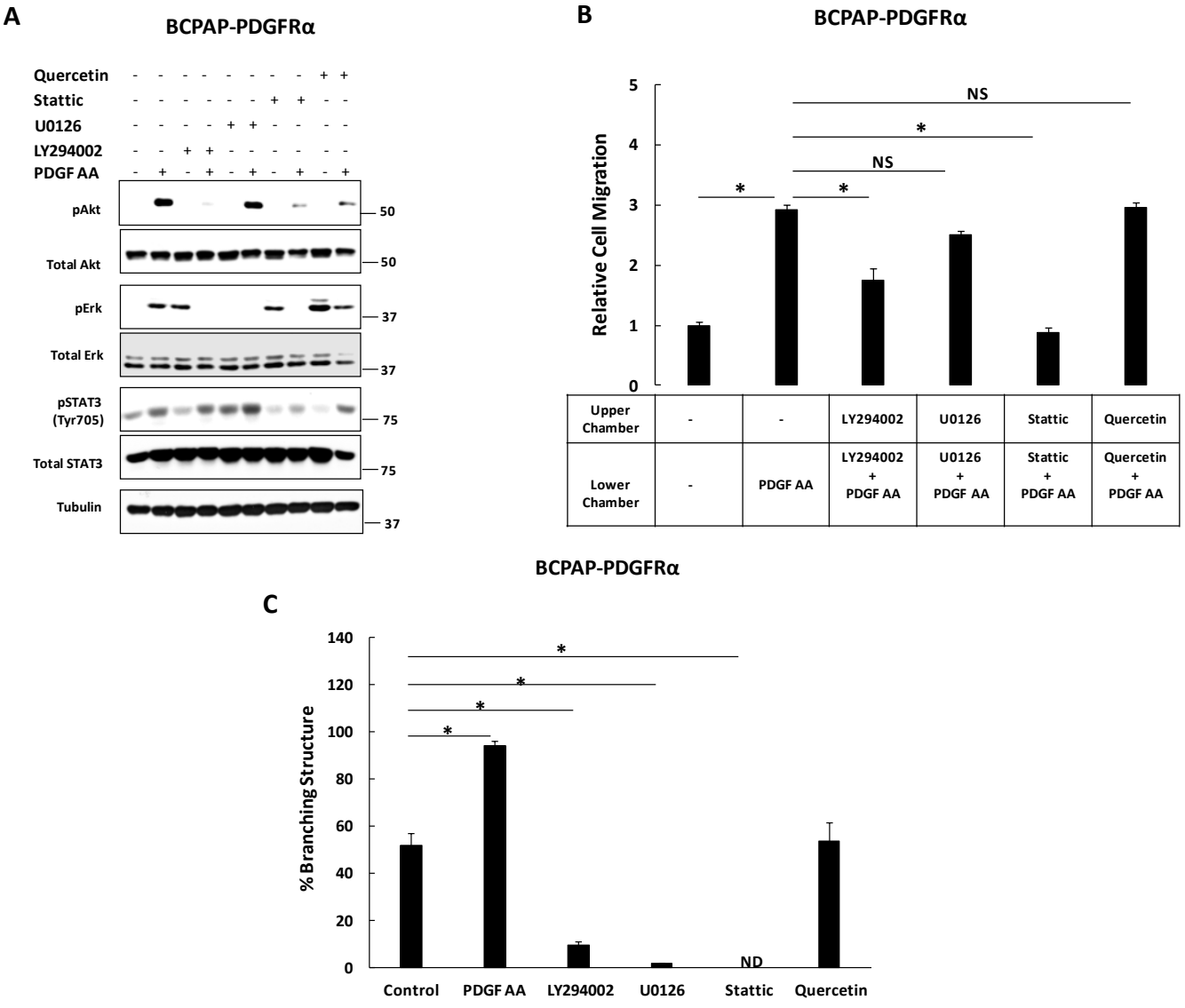

A
BCPAP-PDGFRα
Quercetin
Stattic
U0126
LY294002
PDGF AA
pAkt
Total Akt
pErk
Total Erk
pSTAT3 (Tyr705)
Total STAT3
Tubulin
50
50
37
37
75
75
37
B
BCPAP-PDGFRα
Relative Cell Migration
NS
*
NS
*
*
Upper Chamber
Lower Chamber
LY294002 + PDGF AA
U0126 + PDGF AA
Stattic + PDGF AA
Quercetin + PDGF AA
C
BCPAP-PDGFRα
% Branching Structure
ND
Control
PDGF AA
LY294002
U0126
Stattic
Quercetin

Figure 4.5: Effects of inhibiting signaling pathways on the PDGFRα-associated phenotypes To assess the relative roles of the Erk/MAPK, PI3K/Akt, STAT3 , and Wnt/βcatenin pathways in establishing the PDGFRα-associated phenotypes, these pathways were pharmacologically blocked with U0126 (10 μM), Ly294002 (10 μM), Stattic (2.5 μM) and Quercetin (10 μM), respectively. **(A)** Cells expressing PDGFRα were serum-starved for 24 h, pretreated with the inhibitors for 1 h, and then stimulated with 50ng/ml PDGF-AA for 6 min. Pathway blockade was confirmed by monitoring the phosphorylation levels of Erk, Akt, and STAT3 on western blots. **(B)** PDGFRα-induced migration was quantified in BCPAP-PDGFRα cells following treatment with the inhibitors. **(C)** The proportion of branching structures formed in the presence of the inhibitors was assessed in BCPAP-PDGFRα cells after propagation in 3D culture for 7 days. ND denotes branching structures not detected. Quantitative results **(B and C)** are expressed as means ± SEM, n = 6. Significant differences are indicated by * when $p < 0.05$, NS =Not significant.

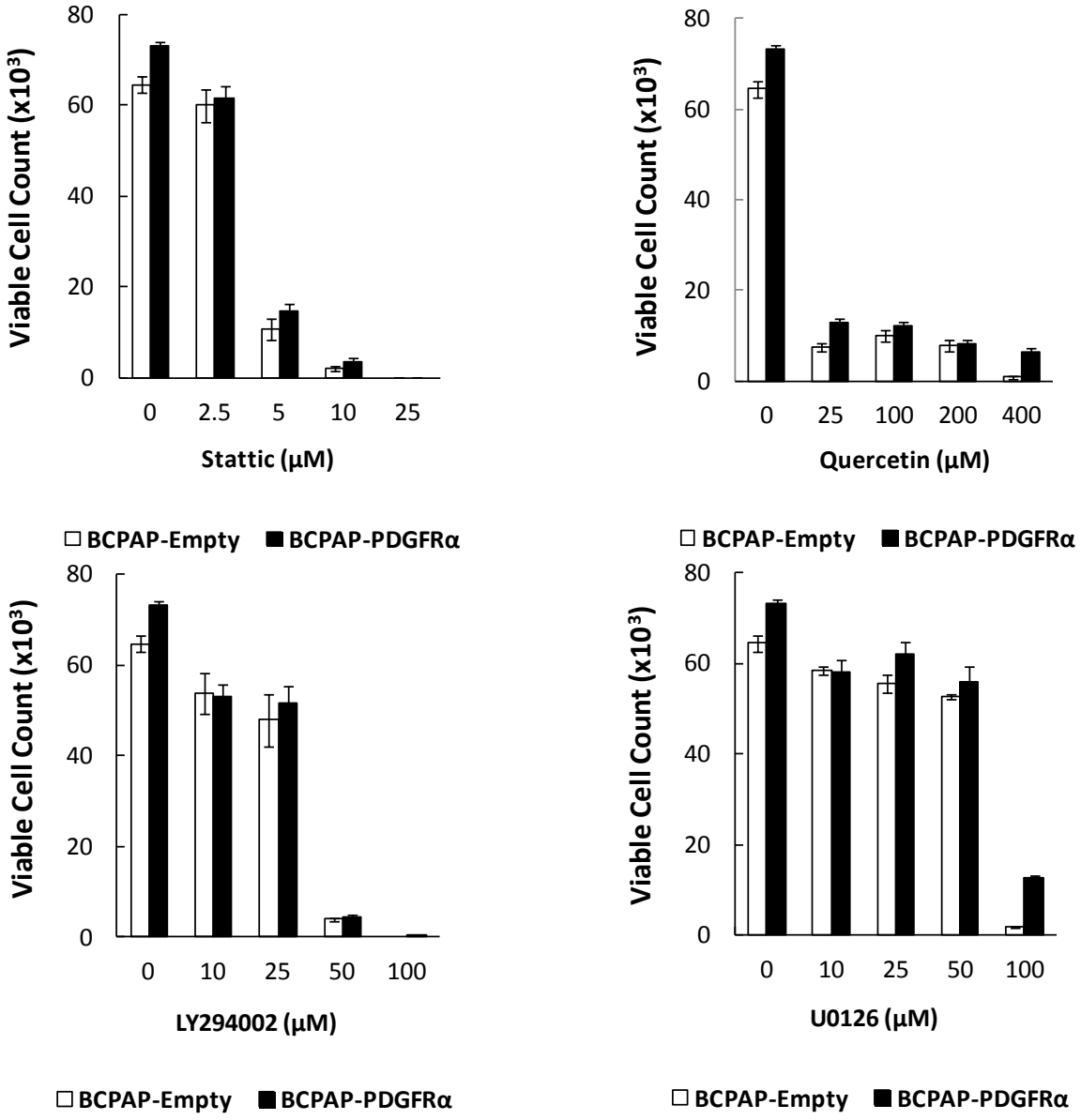


Figure 4.6: PDGFRα expression has no significant effect on the viability of PTC cells in the absence or presence of varied inhibitor concentration. BCPAP cells lacking or expressing the α-receptor were treated with the indicated doses of inhibitors for 24 h and trypan blue exclusion assay was performed. Results are expressed as total number of viable cells (means ± SEM, n = 6)

Given that STAT3 blockade produced a complete morphology reversion with no detectable branched structure, we further checked if this strong inhibitory effect with Stattic was reversible over a concentration range of 0.25 μM, 1 μM and 2.5 μM. Treatment of PDGFRα expressing cells with Stattic was discontinued after 5 days, and cells were allowed to grow for another 5 days. Branching was blocked in a dose-dependent manner; with the highest inhibitory effect seen with 2.5 μM Stattic (**Fig. 4.7A**, lower magnification; **Fig. 4.7B**, higher magnification). After withdrawal, branched structures returned at all concentrations used, however the strongest appearance was observed in cells treated with 0.25 μM Stattic.

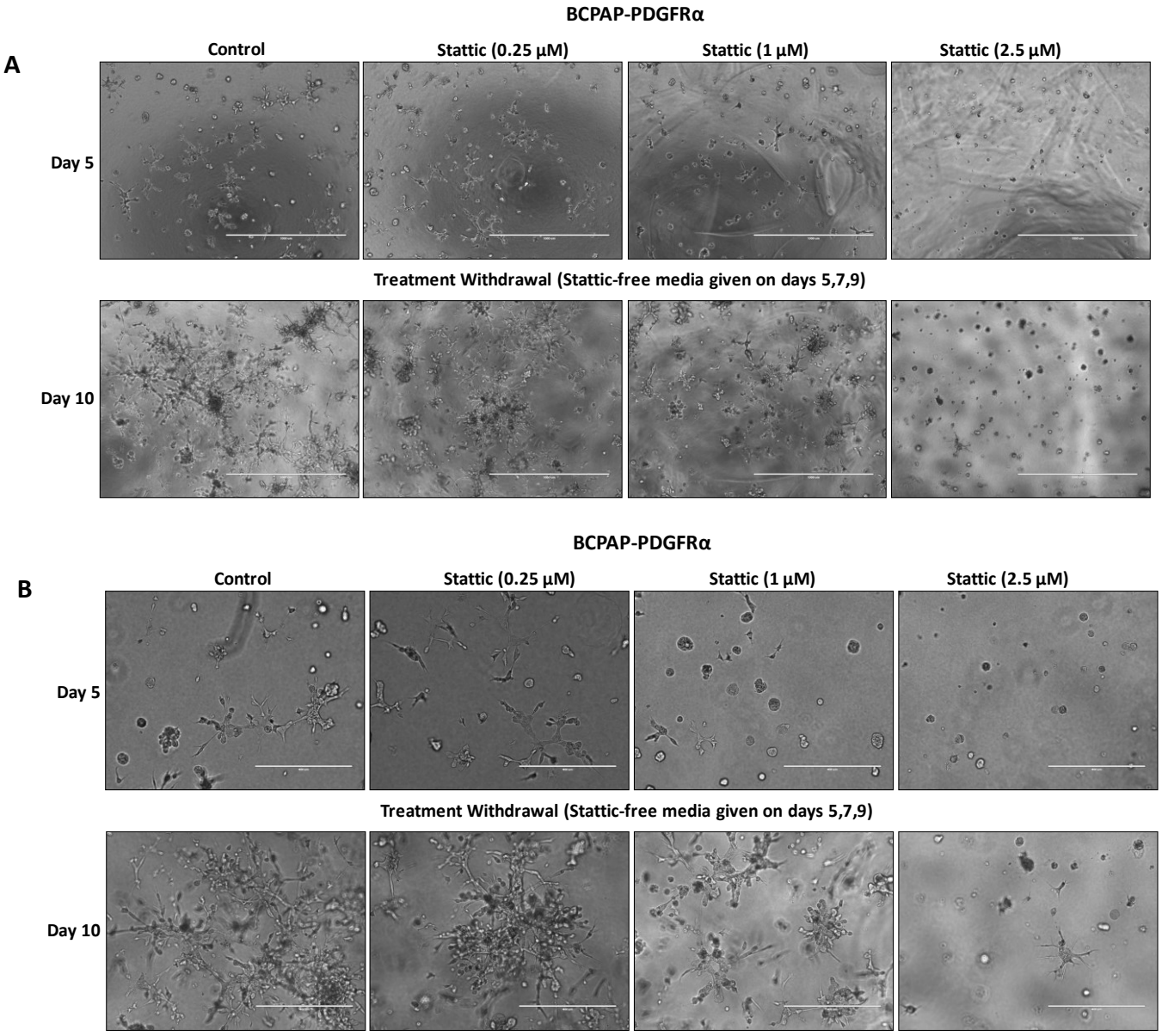

A
BCPAP-PDGFRα
Control
Stattic (0.25 µM)
Stattic (1 µM)
Stattic (2.5 µM)
Day 5
Treatment Withdrawal (Stattic-free media given on days 5,7,9)
Day 10
B
BCPAP-PDGFRα
Control
Stattic (0.25 µM)
Stattic (1 µM)
Stattic (2.5 µM)
Day 5
Treatment Withdrawal (Stattic-free media given on days 5,7,9)
Day 10

Figure 4.7: The inhibitory effect of Statttic on the PDGFRα-induced 3D morphology was assessed for its reversibility over the indicated concentration range. Treatment of PDGFRα expressing cells with Stattic was discontinued after 5 days, and cells were allowed to grow for another 5 days. **(A)** Images were acquired on days 5 and 10 (scale bars; 1000μm). **(B)** Higher magnification of the phase contrast images (scale bars; 400 μm).

PDGFRα Stimulates Nuclear Accumulation of STAT3

Given that STAT3 signaling was crucial for establishing the PDGFRα-associated phenotypes, the relationship between PDGFRα stimulation and STAT3 activity was further established. For STAT3 to regulate the expression of its target genes, its phosphorylation on Tyr705, homodimerization and nuclear translocation are required (22). Since PDGF-AA-induced activation of PDGFRα led to enhanced STAT3 (Tyr705) phosphorylation (**Fig 4.5A**), subsequent nuclear translocation and accumulation of phosphorylated STAT3 (Tyr705) was expected.

To determine if this is the case, starved BCPAP-Empty and BCPAP-PDGFRα cells were treated with PDGF-AA for 20 min, fixed and subjected to immunofluorescence staining for pSTAT3 (Tyr705), PDGFRα and the nucleus. pSTAT3 expression was not detectable in the nucleus and cytoplasm of untreated BCPAP-Empty and BCPAP-PDGFRα cells, but was observed in the nucleus of PDGFRα-expressing cells within 20 min of PDGF-AA stimulation (**Fig. 4.8**). Nuclear and cytoplasmic staining for pSTAT3 (Tyr705) was also absent in BCPAP-Empty cells treated with PDGF-AA.

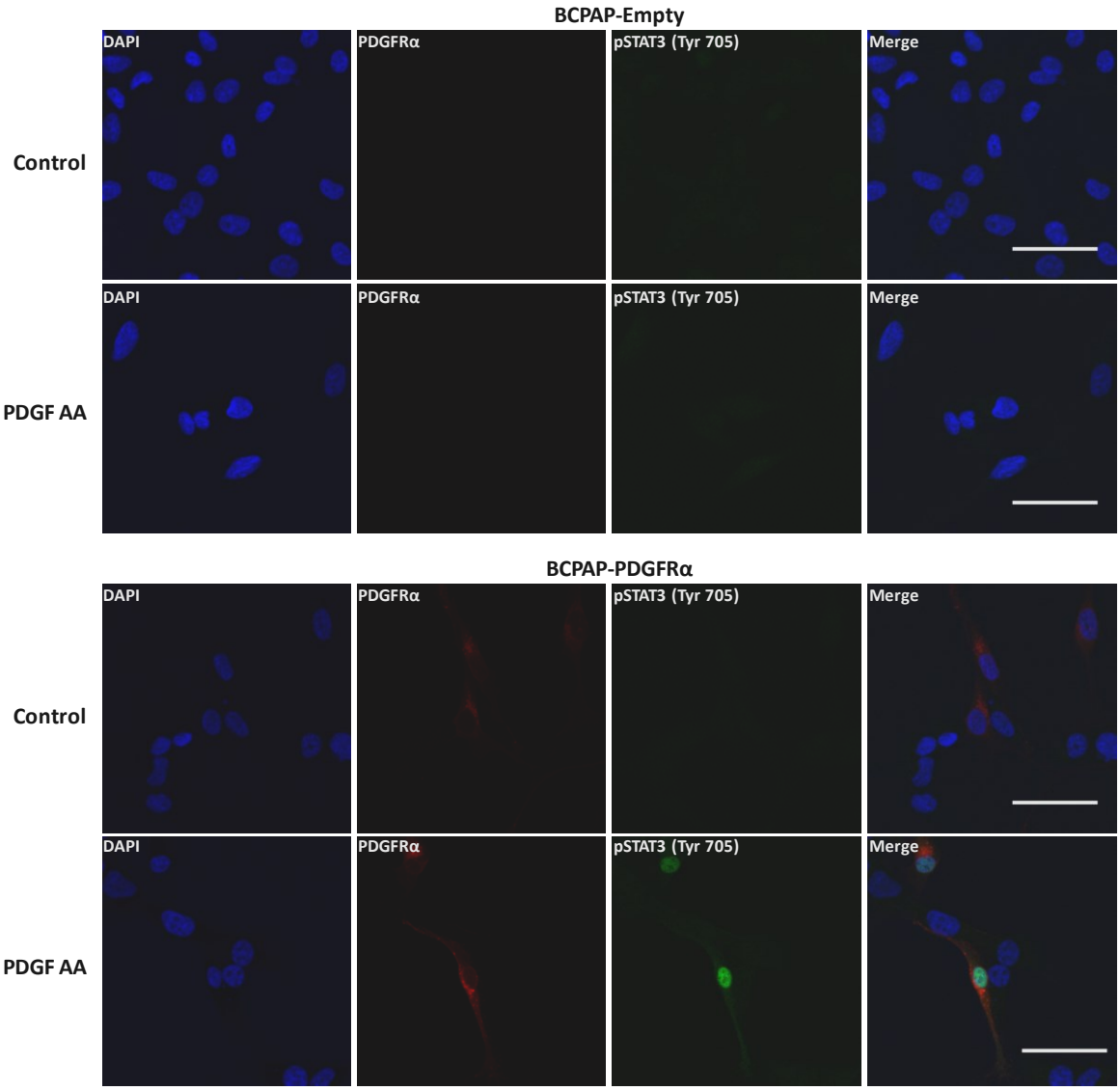
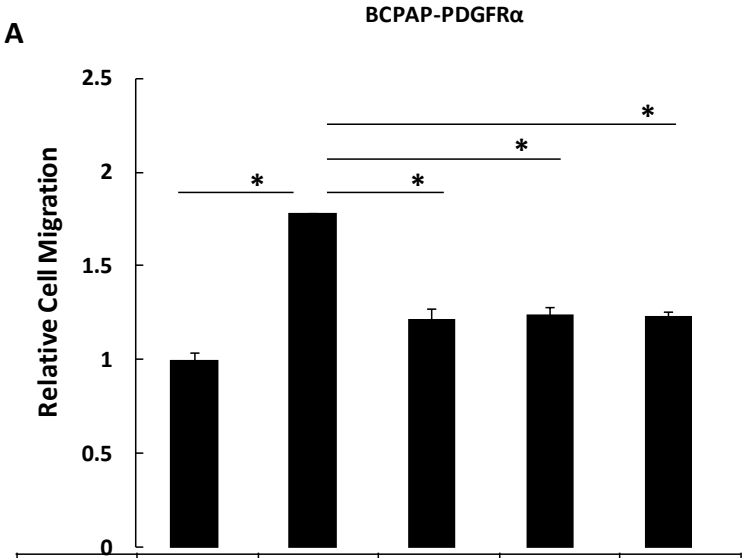


Figure 4.8: PDGFRα activation induces nuclear accumulation of phospho-STAT3 BCPAP-Empty and BCPAP-PDGFRα cells were seeded onto 8-well chamber slides overnight, serum starved for 24 h and treated with or without PDGF-AA (50 ng/ml) for 20 min. The cells were prepared for immunofluorescence staining of PDGFRα (red), pSTAT3 (green), and the nucleus (blue) as described in Materials and Methods. Scale bars are 50 μm.

Inhibition of PDGFRα Activation Impedes Cell Migration in PTC Cells and Abrogates the Transforming Effect of PDGFRα on the 3D Architecture

We further assessed the effect of specifically blocking PDGFRα activity using the TKI, Crenolanib. Crenolanib blocked PDGF-AA-induced migration in serum-starved BCPAP-PDGFRα cells (**Fig. 4.9A**). Disrupting the activation of the PDGFRα abrogates the formation of branched 3D structures in the PDGFRα-expressing cells. With 1 μM Crenolanib, the branched structures were undetectable compared to the untreated and PDGFAA-treated cells in which the percentage of branched structures was 42 ± 3% and 87 ± 5%, respectively (**Fig. 4.9B**). In these PDGFRα-expressing cells, Crenolanib caused a complete architectural transformation to the compact, dense 3D spheres similar to those formed by cells lacking PDGFRα expression (**Fig. 4.9B)**. Next, we checked if the abrogative effect observed with Crenolanib was reversible. BCPAP cells expressing PDGFRα were exposed to different concentrations of Crenolanib for 5 days, after which the treatment was withdrawn. Following the 5-day treatment cells completely reverted to the dense, spherical morphology with the branched structures being undetectable at all Crenolanib concentrations used (**Fig. 4.10A**, lower magnification; **Fig4.10B**, higher magnification). Untreated control cells exhibited the branched morphology as expected. A strong appearance of the branched structures was seen 7 days after the withdrawal of Crenolanib (0.5 μM and 1 μM). At higher concentrations, the compact, spherical morphology induced by Crenolanib treatment was mostly maintained (**Figs. 4.10A** and **4.10B**).

A



Upper Chamber	-	-	Crenolanib (0.1 μM)	Crenolanib (0.5 μM)	Crenolanib (1 μM)
Lower Chamber	-	PDGF AA	Crenolanib (0.1 μM) + PDGF AA	Crenolanib (0.5 μM) + PDGF AA	Crenolanib (1 μM) + PDGF AA

B

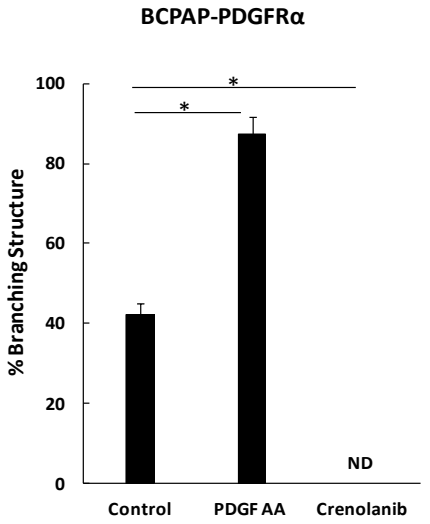


Figure 4.9: Inhibition of PDGFRα activation impedes cell migration in PTC cells and abrogates the transforming effect of PDGFRα on the 3D architecture (A) The migration of serum-starved BCPAP-PDGFRα cells over 4 h in response to the gradient created by PDGF-AA (50 ng/ml) was quantified in the absence or presence of the indicated doses of Crenolanib. Untreated controls without Crenolanib and PDGF-AA were included. Results are means ± SEM, n = 8. Significant differences are indicated by *, $p < 0.05$ **(B)** To assess the effect of blocking PDGFRα activity on the formation of the branched architecture, PDGFRα-expressing cells were grown in 3D culture for 7 days in the absence or presence of 1 μM Crenolanib or 50 ng/ml PDGF-AA. Results are means ± SEM, n = 6. * $p < 0.05$ compared to no treatment control. ND denotes branching structures not detected.

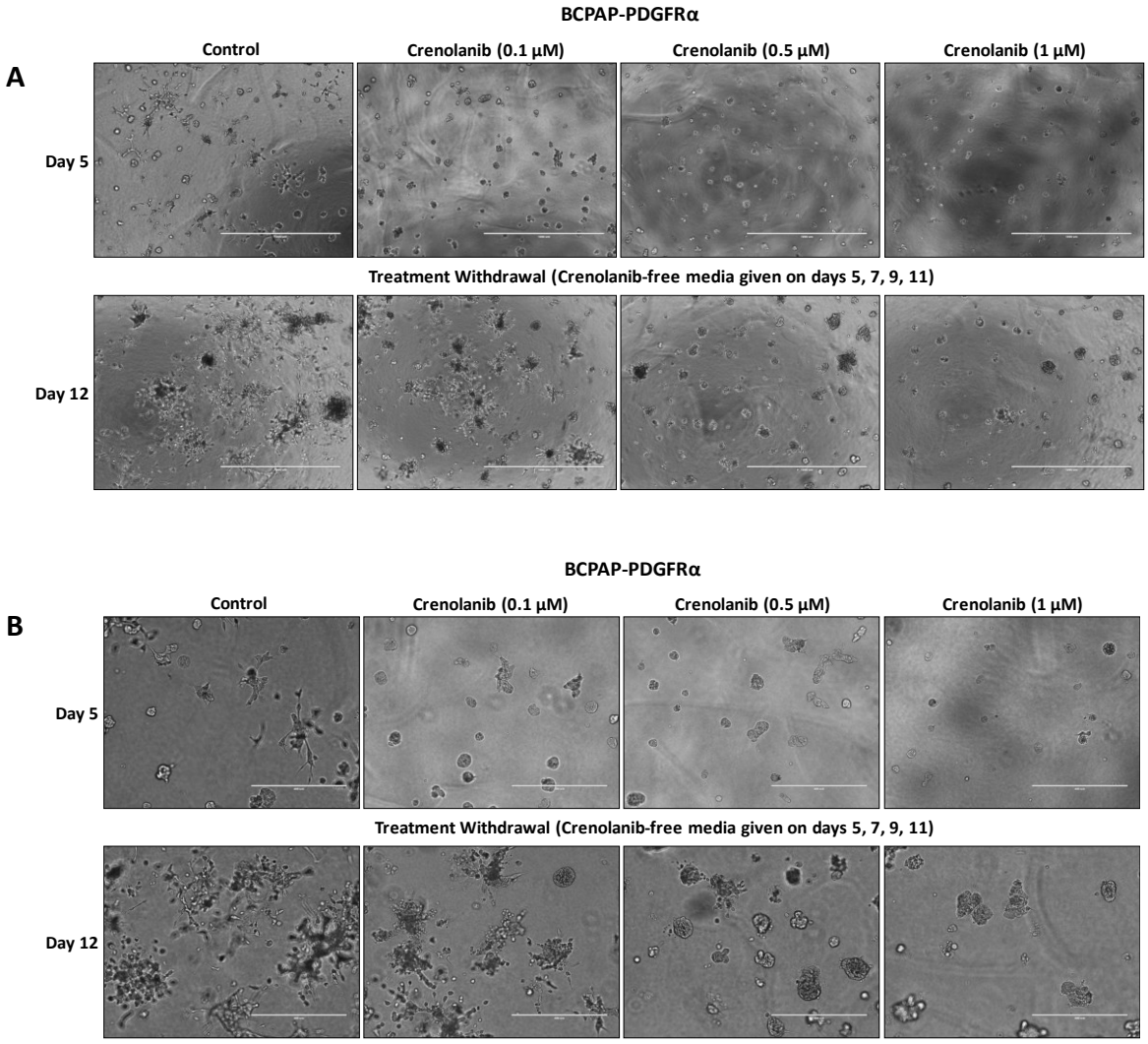


Figure 4.10: The abrogative effect observed with Crenolanib was assessed for its reversibility over the indicted range of concentrations PDGFRα-expressing cells were grown in 3D Matrigel culture in the absence (control) and presence of Crenolanib. Treatment was withdrawn after 5 days of propagation by feeding the cells with Crenolanib-free medium on days 5, 7, 9 and 11. **(A)** Phase contrast images were acquired on days 5 and 12, scale bars are 1000 µm. **(B)** Higher magnification of phase contrast images (Scale bars are 400 µm).

PDGFRα Sensitizes Cells To Epithelial Mesenchymal Transition.

We hypothesised that the invasive-like 3D morphology and increased migratory abilities exhibited by PDGFRα-expressing cells could depend on a PDGFRα–induced epithelial mesenchymal transition (EMT). This is a process through which well restrained, organized epithelial cells, down-regulate their epithelial characteristics to acquire motile, invasive mesenchymal characteristics (23, 24). The expression levels of some EMT-associated markers were assessed in BCPAP-Empty and BCPAP-PDGFRα cells grown as monolayer and treated with PDGF-AA.

There were no significant differences in the expressions of N-cadherin, Twist-1, and vimentin between cells lacking or expressing PDGFRα with or without PDGF-AA stimulation. However, PDGFRα expression dramatically enhanced the protein levels of the transcription factors Snail and Slug, whose levels increased further in response to PDGF-AA stimulation (**Fig. 4.11A)**. Moreover, 1 μM Crenolanib caused a depletion of Snail protein expression in BCPAP-PDGFRα cells in a time-dependent manner, first noticeable after 4 h of treatment (**Fig. 4.11B**). The repressive effect on Slug expression was modest and first evident after 6 h of Crenolanib treatment. Also, since STAT3 signaling seems to be important for the transformations in PDGFRα-expressing BCPAP cells, we examined the effect of blocking STAT3 activation. STAT3 inhibition with Stattic (2.5 μM) repressed Snail protein expression while there was no effect on Slug expression in BCPAP-PDGFRα cells (**Fig. 4.11C**).

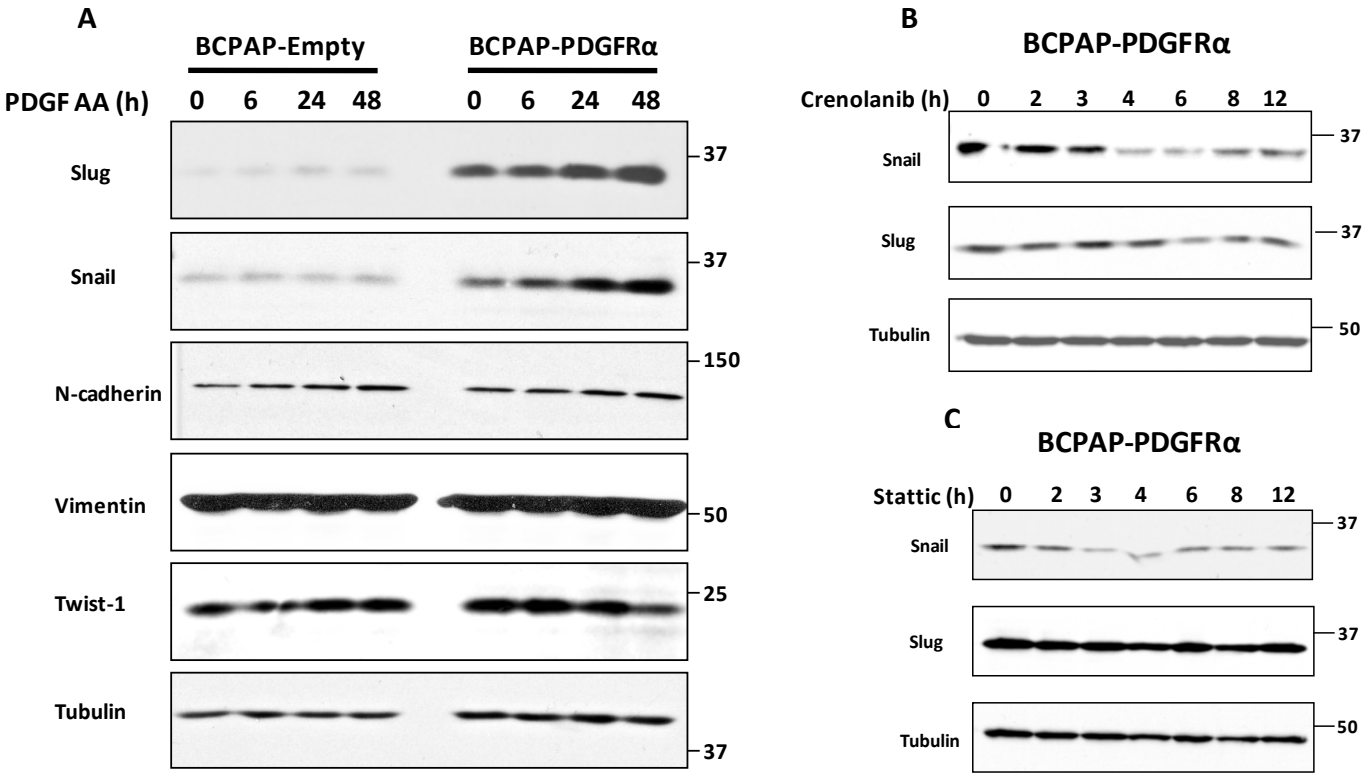

A
BCPAP-Empty
BCPAP-PDGFRα
PDGF AA (h)
0 6 24 48 0 6 24 48
Slug
Snail
N-cadherin
Vimentin
Twist-1
Tubulin
37
37
150
50
25
37
B
BCPAP-PDGFRα
Crenolanib (h) 0 2 3 4 6 8 12
Snail
Slug
Tubulin
37
37
50
C
BCPAP-PDGFRα
Stattic (h) 0 2 3 4 6 8 12
Snail
Slug
Tubulin
37
37
50

Figure 4.11: PDGFRα sensitizes cells to epithelial mesenchymal transition (A) BCPAP cells lacking and expressing PDGFRα were grown as monolayers, serum-starved for 24 h, and stimulated with 50 ng/ml PDGF-AA for the indicated duration. Changes in the expression of EMT–associated makers, Slug, Snail, N-cadherin, Vimentin, and Twist-1 were monitored by the Western blotting with tubulin as the loading control. **(B)** BCPAP- PDGFRα cells were serum-starved for 24 h and exposed to 1 μM Crenolanib for the times indicated. Cell lysates were probed for Snail and Slug expression. **(C)** Similar experiments were performed using 2.5 μM Stattic to block STAT3 activation and the expression of Snail and Slug was monitored. The results are representative of 3 independent experiments.

Crenolanib Abrogates the PDGFRα-induced Accumulation of F-actin Associated with Invadopodia Formation

EMT is believed to be a prerequisite for cancer cell dissociation, invasion, and metastasis. A major event in this process is the reorganization of the cytoskeletal architecture resulting in the formation of actin-rich membrane protrusions, which facilitate directional cell migration (23, 24). There is mounting evidence that these protrusions, which are commonly referred to as invadopodia in cancer cells, play important roles in the metastatic cascade (11, 25-28). Having established a link between PDGFRα activation and the expression of EMT associated markers, Slug and Snail, we determined if the protrusions observed with PDGFRα expression and activation exhibit components of invadopodia.

BCPAP cells lacking or expressing PDGFRα were grown in 3D culture and the accumulation of F-actin, a major structural component of mature invadopodia (29) was examined by immunofluorescence. Relative to cells lacking PDGFRα, increased F-actin staining was observed with the formation of branched structures when PDGFRα was expressed and activated with PDGF-AA (**Fig. 4.12A**). The rich expression of F-actin in the protrusions strongly indicates they could be invadopodia. It is noteworthy that strong PDGFRα expression was seen mostly at the tips of the protrusions (white arrows; **Fig. 4.12A**). This provides further evidence for the involvement of PDGFRα in initiating the formation of the invadopodia-like structures and the consequent profound morphological transformation.

To explore this hypothesis further, we checked if PDGFRα inhibition with Crenolanib affects F-actin expression. Relative to untreated and PDGF-AA-treated PDGFRα-expressing cells; those treated with Crenolanib displayed a large reduction in F-actin staining intensity as they lost the invadopodia-like projections (**Fig. 4.12A**). A significant loss in PDGFRα expression was also observed with Crenolanib treatment. A similar phenomenon was seen in primary cells cultured from the metastatic lymph node (MLN) deposits of a papillary thyroid tumor**.** Native PDGFRα expression in these primary cells was suppressed following their exposure to 1 μM Crenolanib for 72 h (**Fig. 4.12B)**. A comparable finding was reported in glioblastoma cancer stem cells where treatment with Crenolanib decreased PDGFRα protein expression in some cells and abolished it in others (30).

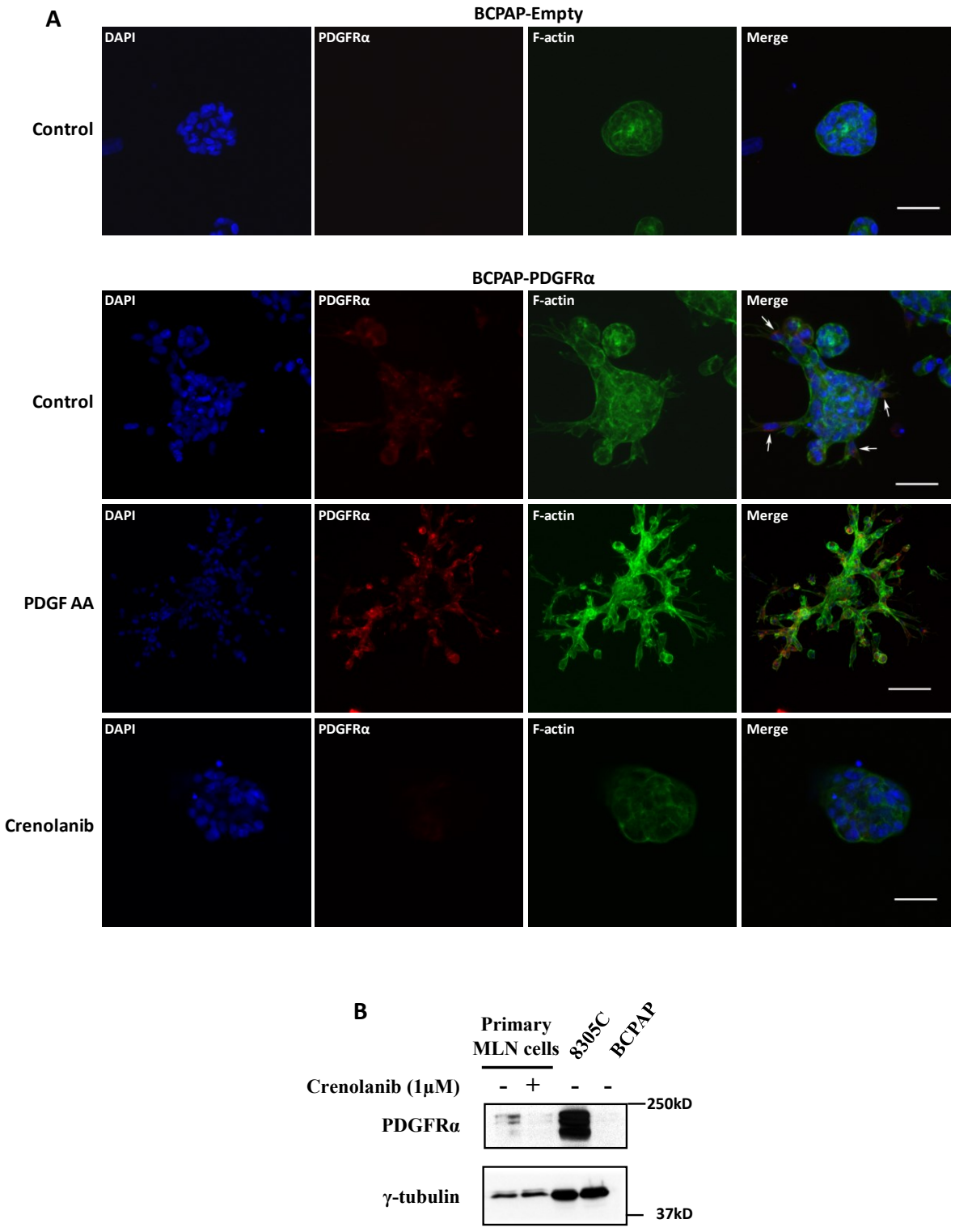
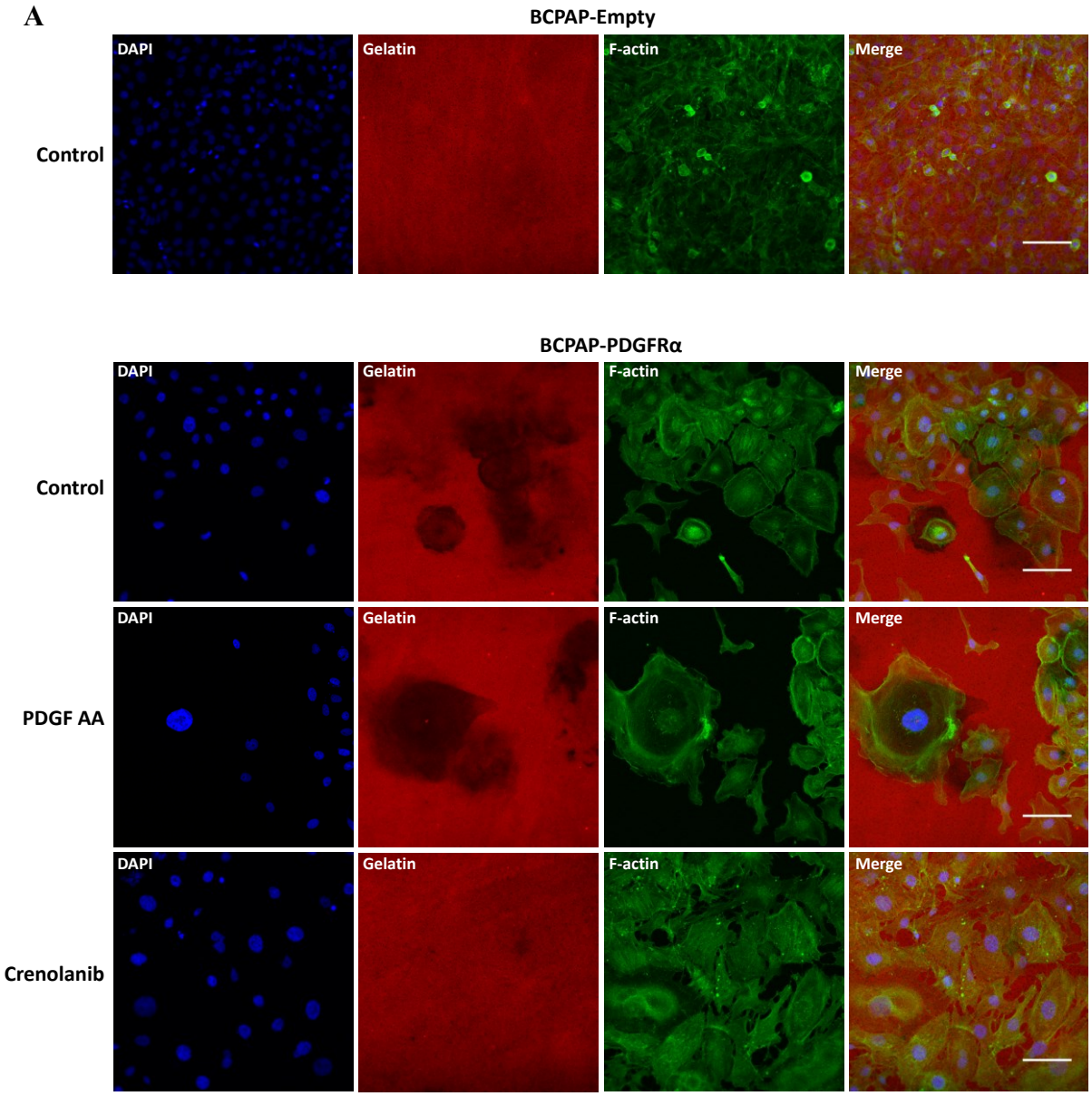

A
BCPAP-Empty
DAPI
PDGFRα
F-actin
Merge
Control
BCPAP-PDGFRα
Control
PDGF AA
Crenolanib
B
Primary MLN cells
8305C
BCPAP
Crenolanib (1μM)
- + - -
PDGFRα
250kD
γ-tubulin
37kD

Figure 4.12: Crenolanib abrogates the PDGFRα-induced accumulation of F-actin associated with invadopodia formation. **(A)** BCPAP cells lacking or expressing PDGFRα were grown in 3D culture in the absence or presence of 1μM Crenolanib or 50 ng/ml PDGF-AA. Immuno-fluorescent staining of PDGFRα (red), F-actin (green) and DAPI (blue) was performed after 7 days in culture. Strong PDGFRα expression was seen at the tips of the protrusions (white arrows). Scale bars are 50 μm**.** **(B)** PDGFRα protein expression in primary cells derived from PTC metastatic lymph nodes (MLN), which were treated with 1 μM Crenolanib for 72 h. 8305C and BCPAP cells served as positive and negative controls respectively, while tubulin expression served as the loading control.

PDGFRα Promotes Gelatin Degradation in PTC Cells

To establish whether the PDGFRα-induced protrusions are functional invadopodia, BCPAP-Empty and BCPAP-PDGFRα cells were assessed for their gelatinase ability. As shown by the immunofluorescent images, gelatin degradation was seen in BCPAP-PDGFRα cells while BCPAP-Empty cells showed no gelatinase ability (**Fig. 4.13A**). The degradation area increased by 4-fold when BCPAP-PDGFRα cells were treated with PDGF-AA compared to the untreated (control) cells (**Fig. 4.13B**). Crenolanib, almost completely abolished the degradative ability of BCPAP-PDGFRα cells, and the degradation area significantly reduced by ~8 fold relative to the control cells (**Fig. 4.13B**). As delineated by F-actin staining, BCPAP-PDGFRα cells with high gelatinase activity seemed to be larger than the BCPAP-Empty cells with no degradative activity (**Fig. 4.13A**).


A
BCPAP-Empty
DAPI
Gelatin
F-actin
Merge
Control
BCPAP-PDGFRα
DAPI
Gelatin
F-actin
Merge
Control
DAPI
Gelatin
F-actin
Merge
PDGF AA
DAPI
Gelatin
F-actin
Merge
Crenolanib

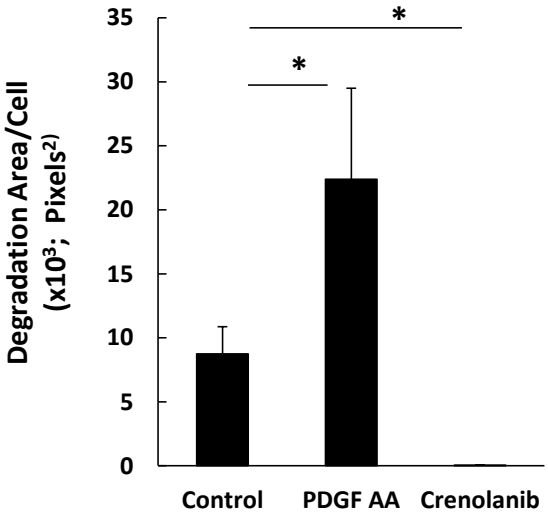


Figure 4.13: PDGFRα activation increases invadopodia degradation activity. (A) BCPAP-Empty and BCPAP-PDGFRα were plated on cy3-conjugated gelatin and cultured with or without PDGF-AA and Crenolanib for 4 days. Cells were stained for F-actin (green) and nuclei (blue) and imaged at 20X objective magnification at 5 fields of view per well. Representative image sets are shown for all treatments (scalebar = 100 μm). Dark patches on cy3-conjugated gelatin (red) indicate areas of degradation. **(B)** Quantification of mean degradation area per cell as described in the Materials and Methods. *, $p < 0.05$

Taqman Low density Array (TLDA) Analysis

After confirming of that the invadopodial structures are functional, we further outlined the gene profile that accompanies the PDGFRα-induced biogenesis of these structures in PTC cells. Using TLDA cards customized for assessing the expression of 188 gene-set associated with tumor metastasis (**Table 4.1**), we compared BCPAP-PDGFRα and BCPAP-Empty cells propagated in 3D culture for 7 days, in the presence of PDGF-AA (50ng/ml). Out of the genes significantly altered between BCPAP-PDGFRα and BCPAP-Empty cells, seven were identified with major variations in expression. They include *SPP1, CDH1 and LPAR4, LPAR1, KIT, ESR1, and BCL2* (**Fig. 4.14)**.

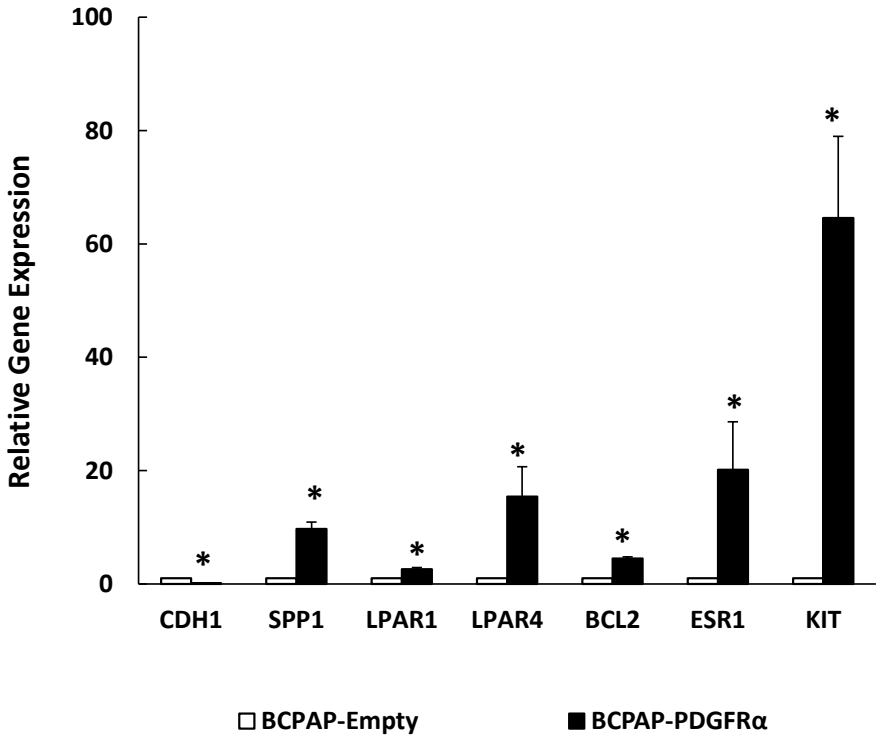


Figure 4.14: TaqMan Low-Density Array analysis of cells with (BCPAP-PDGFRα) and without (BCPAP-Empty) functional invadopodia. Gene expression analysis was performed following a 7-day propagation in Matrigel. Results show seven significantly altered genes selected for future investigation. Values relative to GAPDH; Data are presented as mean ± SEM. *, $p < 0.05$

4.3 Discussion

In Chapter 4, we reveal that the interesting association of PDGFRα with nodal metastases seen in clinical specimens, may be driving a remarkable phenotypic transformation embodied by the epithelial to mesenchymal transition and formation of invadopodia in PTC cells. Moreover, we document a very strong role for multiple signaling pathways driving this more aggressive phenotype in our biochemical studies.

First, we demonstrated the PDGFRα-dependent expression of EMT-associated transcription factors, Snail and Slug in PTC cells. The EMT is a well-regulated physiological process in embryogenesis where it promotes the mesenchymal cell development. However, this process is now commonly accepted as a major pathologic mechanism in epithelial tumor progression, local invasion, and metastasis (31). During the EMT, non-motile, polarized epithelial cells shed their intracellular adhesion molecules to become individual, non-polarized, motile mesenchymal cells. Consequently, cancer cells acquire migratory and invasive phenotypes, which involve a dramatic reorganization of the actin cytoskeleton and the concomitant formation of membrane protrusions required for invasive growth and metastasis (31).

The up-regulation of Snail and Slug with corresponding increases in aggressiveness has been reported in many human malignancies (32-34), including PTCs where they were detected in lymph node metastases (35). The gene expression changes, which provoke the behavioral switch from the epithelial to the mesenchymal phenotype are mediated by regulators like Snail and Slug, whose expression is

activated early in EMT. However, variations in EMT-associated gene expression profiles depend on the cell type as well as the extent of progression towards the mesenchymal differentiation (23). Hence, the nature of the cells used here and the extent of their transition into the mesenchymal phenotype, might explain why the expression of the other EMT markers, N-cadherin, Twist-1 and vimentin, was not altered in the PDGFRα-expressing BCPAP cells.

Also believed to be an ensuant response to the epithelial-mesenchymal-transition process in cancer cells, is the formation of invadopodia (11, 25-28). For the first time, provided here is the evidence of a link between PDGFRα signaling and functional invadopodia production in PTC cells. Invadopodia are thought to be aberrant derivatives of podosomes (in immune cells and neurons) usurped by cancer cells for invasion and migration during metastasis (11, 25, 27). They are vital for multiple steps in the metastatic cascade, including local invasion into the stromal tissues at the primary tumor, intravasation into the vasculature, extravasation at distant sites, and colonization of distant organs (36).

The migration of cells undergoing EMT depends on the structural integrity of the invadopodial protrusions following cytoskeletal rearrangement, a process hallmarked by F-actin polymerization (11). In our study, we show that the protrusions were enriched with F-actin, providing substantial evidence that PDGFRα induces cytoskeletal remodelling in PTC cells undergoing EMT. In addition, PDGFRα-expressing cells displayed increased motility in response to a PDGF-AA gradient, indicating a migratory role for the invadopodia-like projections seen in 3D culture.

This suggestion is founded on a previous observation that invadopodia are also involved in signal sensing and directional protrusion during chemotaxis (37). Similar to our findings, Gotzmann *et al.*, (38) also reported that the PDGF-AA/PDGFRα signaling axis promotes tumor progression by enhancing the motility of hepatocytes undergoing EMT.

We went on to characterise the PDGFRα-induced protrusions as functional invadopodia, adjudged by their gelatinase activity. Visualization of invadopodia-mediated ECM degradation by microscopy using fluorescently-labeled matrix proteins has emerged as the most prevalent technique for evaluating matrix degrading capability and cellular invasiveness (39). Overexpression of PDGFRα and its activation in PTC cells drastically increased the ability of the cells to degrade cy3-labeled gelatin matrix. PDGFRα-deficient cells displayed null gelatinase ability. Tumor cells leverage the aptitude of the invadopodial protrusions for ECM degradation to overcome the physical barriers presented by the basement membrane, the interstitial matrix, and the endothelial cells during metastasis (40). The ultimate function of invadopodia is to recruit various matrix proteases to cell-ECM focal contacts for matrix degradation (36). Numerous *in vitro* observations of enzymatic ECM degradation by invadopodia-forming cells have been made in cultures of melanoma, breast, prostate, as well as head and neck squamous cancer cells (41-43). Similar to our findings, a positive association between EMT transcriptional regulators, Twist1 and Snail, as well as PDGFRα expression was shown to be necessary for invadopodia development in breast cancer cells (11).

Given that PDGFRα induces a degradative, migratory phenotype in PTC cells, we investigated the downstream effectors of its pro-metastatic cues. PI3K/Akt, MAPK/Erk, and STAT3 were activated by PDGFRα in PTC cells. Blocking these pathways, especially STAT3, with pharmacological agents disrupted the invadopodia-like formations in PDGFRα-expressing cells, and reduced the number of migrating cells in 2D culture. These results strongly implicate the STAT3 pathway as the major effector of the pro-metastatic signals from PDGFRα, while the PI3K/Akt and MAPK/Erk pathways played lesser or adjunct roles. Our finding mirrors previous reports of constitutive STAT3 activation in response to aberrant upstream tyrosine kinase activities in a broad spectrum of human cancers (44). More specifically, histological analysis showed increased expression and activation of STAT3 in the nodal deposits of metastasis PTCs (15). These results directly challenge the canonical description of PTC as a MAPK/Erk-driven malignancy, and clearly demonstrate a vital role for the STAT3 and AKT pathways in driving this aggressive disease phenotype.

In vivo evidence from animal models showing that invadopodia are crucial for successful metastasis have also been documented (25, 45). Thus, our results suggest that PDGFRα promotes the biogenesis of functional invadopodia in PTC cells, as part of its metastasis-driving mechanism. On this note, it became necessary to identify supporting participants in this PDGFRα-driven process of invadopodia generation, so that a broader understanding of this pro-metastasis process can emerge. Accordingly, a comparative qPCR-based gene expression analysis between BCPAP-PDGFRα and

BCPAP-Empty cells was performed. Of all 188 metastasis-related genes assessed, seven were identified with major variations in expression. *SPP1*, *LPAR4*, *LPAR1*, *KIT*, *ESR1*, and *BCL2* were upregulated, while *CDH1* was downregulated in the PDGFRα-positive cells.

E-cadherin is the major component of epithelial adherens junctions, which along with tight junctions mediate cell-cell adhesion (31, 46). As observed in BCPAP-PDGFRα cells, downregulation of the E-cadherin gene (*CDH1*) correlates with tumor invasiveness, metastasis and patient mortality in most epithelial cancers (46).

SPP1 encodes for a protein commonly called secreted phosphoprotein-1 (SPP1) or ostepontin (OPN) (47). SPP1 is a phosphorylated glycoprotein secreted by activated macrophages, leukocytes, and T lymphocytes. In line with our observation, overexpression of SPP1 increased invadopodia formation and gelatinolytic activity via integrin αvβ3 signaling in prostate cancer cells. This may be attributed to the known ability of SPP1 to induce the expression and activity of metalloproteinases, such as MMP9 in human chondrosarcoma cells (48), and MMP2 in human GCT23 giant cell tumor cells (49). While there is no evidence linking PDGF signaling to SPP1 expression/activity in any tumor type, this relationship is open for further validation in PTC cells. It is worth mentioning though, that PDGF-BB-induced autocrine expression of SPP1 has been shown to play a major role in the migration of normal smooth muscle cells (50).

Genes encoding two receptors (LPAR4 and LPAR1) associated with autotaxin (ATX) signaling were enriched in invadopodia-forming (BCPAP-PDGFRα) cells. ATX catalyses the production of lyosophosphatidate (LPA), which is responsible for the majority of ATX effects (51). ATX, through LPA, plays a role in embryogenesis, angiogenesis, wound healing, and tissue remodeling (52). The physiological regulation of these processes is compromised in many cancers, where high ATX expression and activity increases tumor aggressiveness, metastasis, angiogenesis, and chemoresistance (51-57). The autotaxin gene is among the top 40 upregulated genes in metastatic colon, breast and prostate cancers (58). LPA drives cancer cell division, survival and migration by signaling through six G-protein-coupled-receptors (LPAR1-6; refs. 54-56). LPA also promotes cancer cell resistance to chemotherapy and radiotherapy (54, 59).

This result corroborates reports from a parallel study within our laboratory, in which the ATX/LPA axis is being assessed for its role in thyroid and breast cancers (52, 60). The authors showed that increased expression of ATX, LPA and inflammatory cytokines is prognostic of papillary thyroid tumors and their metastasis (60). Even more, the ATX/LPA/inflammatory axis was shown to be an effective therapeutic target in thyroid and breast cancers (52, 60). Blocking ATX activity repressed tumor growth in mouse models of breast and thyroid cancer, and decreased levels of PDGF-AA and lymphangiogenic VEGF were observed in the tumors (52, 60). Subsequent lung metastasis was also antagonized in the breast cancer mouse model with the ATX inhibition (52).

Targeted inhibition of PDGFRα as a strategy for treating cancers with aberrant expression of this receptor has become the focus of many clinical studies and trials (17, 18, 61-65). Efforts to target PDGFRα have yielded the development of potent inhibitors of its activity such as imatinib, sunitinib, sorafenib, pazopanib and nilotinib. However, these TKIs lack specificity and they act on a spectrum of tyrosine kinases. For instance, in addition to inhibiting PDGFRα, sunitinib inhibits PDGFRβ and the vascular endothelial growth factor receptors (2). Crenolanib, a novel TKI, which targets and inhibits PDGFRα activity with high specificity, has been tested for its therapeutic efficacy in many human tumors (17, 18, 61). Here, we have shown that the pharmacological inhibition of PDGFRα with Crenolanib effectively abrogated the invasive and migratory behavior associated with this receptor.

Overall, this Chapter provides a mechanistic explanation that PDGFRα creates an invasive cellular phenotype and a dramatic increase in the ability to of the cells to migrate, as a consequence of epithelial to mesenchymal transition. Consistent with this hypothesis, Crenolanib, a highly specific inhibitor of PDGFRα, suppressed the levels of Snail and Slug and abrogated the aggressive phenotypes associated with PDGFRα. This is the first evidence suggesting that inhibiting the activation of PDGFRα and the EMT regulators, Snail and Slug, could provide a potentially beneficial treatment for metastatic PTC patients whose tumors over-express this receptor.

Table 4.1: The 192 gene-set duplicated on the 384-well TLDA, out of which 188 genes encode proteins relevant in mechanisms which drive tumor growth and metastasis; 4 replicates of the GAPDH gene were included to standardize the expression of the target genes. RQ: relative quantitation of gene (BCPAPPDGFRα/ BCPAP-Empty); ND: not detectable

Gene	Description	RQ
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	0.6
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	1.2
AKT1	v-akt murine thymoma viral oncogene homolog 1	1.0
AKT2	v-akt murine thymoma viral oncogene homolog 2	0.7
AKT3	v-akt murine thymoma viral oncogene homolog 3	1.2
ALDH1A3	aldehyde dehydrogenase 1 family, member A3	1.3
ANGPT1	angiopoietin 1	1.3
APAF1	apoptotic peptidase activating factor 1	0.9
ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7	1.3
ATF2	activating transcription factor 2	0.9
ATG12	ATG12 autophagy related 12 homolog (S. cerevisiae)	1.1
ATG4B	ATG4 autophagy related 4 homolog B (S. cerevisiae)	2.7
AURKB	aurora kinase B	1.7
AXL	AXL receptor tyrosine kinase	0.8
BAD	BCL2-associated agonist of cell death	1.3
BAK1	BCL2-antagonist/killer 1	1.5
BAX	BCL2-associated X protein	1.2
BBC3	BCL2 binding component 3	1.1

BCL2	B-cell CLL/lymphoma 2	4.5
BCL2L1	BCL2-like 1	0.8
BCL2L11	BCL2-like 11 (apoptosis facilitator)	0.8
BECN1	beclin 1, autophagy related	0.7
BID	BH3 interacting domain death agonist	0.9
BIK	BCL2-interacting killer (apoptosis-inducing)	0.7
BIRC2	baculoviral IAP repeat containing 2	0.7
BIRC5	baculoviral IAP repeat containing 5	1.2
BIRC6	baculoviral IAP repeat containing 6	1.0
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	1.2
BRCA1	breast cancer 1, early onset	1.5
BRCA2	breast cancer 2, early onset	1.3
CALR	calreticulin	0.9
CAMK2N1	calcium/calmodulin-dependent protein kinase II inhibitor 1	1.1
CASP3	caspase 3, apoptosis-related cysteine peptidase	1.2
CASP8	caspase 8, apoptosis-related cysteine peptidase	1.2
CASP9	caspase 9, apoptosis-related cysteine peptidase	1.0
CAV1	caveolin 1, caveolae protein, 22kDa	1.3
CCNB1	cyclin B1	1.0
CCND1	cyclin D1	0.8
CCR6	chemokine (C-C motif) receptor 6	0.6
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	0.2
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	1.1
CDK4	cyclin-dependent kinase 4	1.6
CDKN1A	cyclin-dependent kinase inhibitor 1A	0.8

CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.3
CDKN2A	cyclin-dependent kinase inhibitor 2A	1.2
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	2.3
CDKN2D	cyclin-dependent kinase inhibitor 2D	0.9
CHEK2	checkpoint kinase 2	1.0
COL1A2	collagen, type I, alpha 2	6.2
COL3A1	collagen, type III, alpha 1	7.0
CREB1	cAMP responsive element binding protein 1	0.7
CREBBP	CREB binding protein	1.1
CSF1R	colony stimulating factor 1 receptor	0.9
Dec-01	deleted in esophageal cancer 1	0.4
DPP4	dipeptidyl-peptidase 4	4.6
DSC1	desmocollin 1	ND
DSG1	desmoglein 1	0.4
DSP	desmoplakin	1.0
E2F1	E2F transcription factor 1	0.8
ENAH	enabled homolog (Drosophila)	1.4
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	1.2
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	1.2
ERC1	ELKS/RAB6-interacting/CAST family member 1	1.1
ESR1	estrogen receptor 1	20.1
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	0.9
EZR	ezrin	0.8
FAS	Fas (TNF receptor superfamily, member 6)	0.7

FASLG	Fas ligand (TNF superfamily, member 6)	8.1
FGF1	fibroblast growth factor 1 (acidic)	1.6
FGFR1	fibroblast growth factor receptor 1	0.9
FGFR2	fibroblast growth factor receptor 2	1.2
FGFR3	fibroblast growth factor receptor 3	1.0
FGFR4	fibroblast growth factor receptor 4	1.4
FLT1	fms-related tyrosine kinase 1	0.0
FLT4	fms-related tyrosine kinase 4	ND
FOXA1	forkhead box A1	1.9
FOXE1	forkhead box E1 (thyroid transcription factor 2)	0.8
FZD2	frizzled family receptor 2	1.7
FZD7	frizzled family receptor 7	1.0
GATA3	GATA binding protein 3	1.2
GNG11	guanine nucleotide binding protein (G protein), gamma 11	1.1
GRB7	growth factor receptor-bound protein 7	0.9
GSK3B	glycogen synthase kinase 3 beta	1.0
HHEX	hematopoietically expressed homeobox	1.4
HPRT1	hypoxanthine phosphoribosyltransferase 1	1.2
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	0.8
ID1	inhibitor of DNA binding 1	0.8
IGF2R	insulin-like growth factor 2 receptor	0.8
IGFBP3	insulin-like growth factor binding protein 3	1.2
ILK	integrin-linked kinase	1.0
INSR	insulin receptor	1.0
ITGAV	integrin, alpha V	1.2
ITGB1	integrin, beta 1 (fibronectin receptor)	1.5

JAG1	jagged 1	1.2
JUP	junction plakoglobin	1.0
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	64.6
KRT18	keratin 18	1.0
KRT5	keratin 5	ND
KRT8	keratin 8	0.9
LPAR1	lysophosphatidic acid receptor 1	2.6
LPAR2	lysophosphatidic acid receptor 2	0.7
LPAR3	lysophosphatidic acid receptor 3	1.8
LPAR4	lysophosphatidic acid receptor 4	15.4
MAPK1	mitogen-activated protein kinase 1	0.8
MAPK10	mitogen-activated protein kinase 10	5.9
MAPK11	mitogen-activated protein kinase 11	0.5
MAPK6	mitogen-activated protein kinase 6	1.0
MAPK8IP2	mitogen-activated protein kinase 8 interacting protein 2	0.2
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	1.6
MDM2	Mdm2, p53 E3 ubiquitin protein ligase homolog (mouse)	1.1
MET	met proto-oncogene (hepatocyte growth factor receptor)	0.9
MMP2	matrix metallopeptidase 2 (gelatinase A)	0.2
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	**2.0**
MMP9	matrix metallopeptidase 9 (gelatinase B)	1.3
MSN	moesin	0.8
MTOR	mechanistic target of rapamycin	0.6
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1.1
MYL9	myosin, light chain 9, regulatory	1.3

NAIP	NLR family, apoptosis inhibitory protein	1.0
NFE2L2	nuclear factor (erythroid-derived 2)-like 2	1.0
NOTCH1	Notch 1	0.9
OCLN	occludin	0.7
OPA1	optic atrophy 1 (autosomal dominant)	1.2
PAPSS1	3'-phosphoadenosine 5'-phosphosulfate synthase 1	0.9
PAX8	paired box 8	0.9
PDGFA	platelet-derived growth factor alpha polypeptide	0.9
PDGFB	platelet-derived growth factor beta polypeptide	59.2
PDGFD	platelet derived growth factor D	3.2
PDGFRA	**platelet-derived growth factor receptor, alpha**	**2285.5**
PDGFRB	platelet-derived growth factor receptor, beta	1.3
PDK1	pyruvate dehydrogenase kinase, isozyme 1	0.8
PGF	placental growth factor	1.0
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	0.6
PIK3R2	phosphoinositide-3-kinase, regulatory subunit 2 (beta)	1.1
PLD1	phospholipase D1, phosphatidylcholine-specific	1.7
PLD2	phospholipase D2	0.6
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	0.9
PPAP2A	phosphatidic acid phosphatase type 2A	0.8
PPAP2B	phosphatidic acid phosphatase type 2B	1.5
PPAP2C	phosphatidic acid phosphatase type 2C	1.2
PPIA	peptidylprolyl isomerase A	0.7
PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit	1.2
PTEN	phosphatase and tensin homolog	1.1
PTGS2	prostaglandin-endoperoxide synthase 2	0.4

PTK2	PTK2 protein tyrosine kinase 2	1.6
PTK2B	PTK2B protein tyrosine kinase 2 beta	0.7
PVRL1	poliovirus receptor-related 1	1.1
PXN	paxillin	1.5
RASA1	RAS p21 protein activator (GTPase activating protein) 1	1.1
RB1	retinoblastoma 1	1.2
RET	ret proto-oncogene	ND
RHOA	ras homolog family member A	0.9
ROCK1	Rho-associated, coiled-coil protein kinase 1	1.1
RTN4	reticulon 4	1.7
SLC26A4	solute carrier family 26, member 4	0.7
SLC39A6	solute carrier family 39 (zinc transporter), member 6	1.0
SLC5A5	solute carrier family 5 (sodium iodide symporter), member 5	ND
SMAD2	SMAD family member 2	0.9
SNAI1	snail homolog 1 (Drosophila)	1.4
SNAI3	snail homolog 3 (Drosophila)	0.6
SOX10	SRY (sex determining region Y)-box 10	ND
SOX2	SRY (sex determining region Y)-box 2	1.1
SPP1	secreted phosphoprotein 1	9.7
STAT3	signal transducer and activator of transcription 3	0.7
TCF3	transcription factor 3	1.1
TFF3	trefoil factor 3 (intestinal)	ND
TFPI2	tissue factor pathway inhibitor 2	1.5
TG	thyroglobulin	ND
TIMP1	TIMP metallopeptidase inhibitor 1	0.9

TMEM132A	transmembrane protein 132A	1.4
TNFAIP8	tumor necrosis factor, alpha-induced protein 8	1.0
TP53	tumor protein p53	1.6
TSC1	tuberous sclerosis 1	0.9
TTF1	transcription termination factor, RNA polymerase I	0.7
TWIST1	twist homolog 1 (Drosophila)	1.4
TYMP	thymidine phosphorylase	1.2
ULK1	unc-51-like kinase 1 (C. elegans)	1.3
VASP	vasodilator-stimulated phosphoprotein	0.9
VCAN	versican	1.5
VEGFA	vascular endothelial growth factor A	1.6
VEGFC	vascular endothelial growth factor C	1.5
WNT11	wingless-type MMTV integration site family, member 11	ND
WNT3A	wingless-type MMTV integration site family, member 3A	ND
WNT5A	wingless-type MMTV integration site family, member 5A	0.8
WNT5B	wingless-type MMTV integration site family, member 5B	2.4
XIAP	X-linked inhibitor of apoptosis	1.2
ZEB1	zinc finger E-box binding homeobox 1	1.1
ZEB2	zinc finger E-box binding homeobox 2	1.6

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CHAPTER FIVE: PDGFRα INDUCES DEDIFFERENTIATION AND SUPRESSES IODIDE UPTAKE IN PAPILLARY THYROID TUMORS, ALTERING CLINICAL OUTCOMES.

5.1 Introduction

The existence of an intricate link between tumor dedifferentiation and metastasis has been reported in PTC (1-5). Well-differentiated carcinomas often lose epithelial differentiation and metastasize as the disease progresses (6). Indeed, the influence of tumor differentiation on the metastatic process is evident in the tumor grading systems used in surgical pathology (7-9). These systems are grounded in the experience that poorly differentiated tumors are usually more invasive than their differentiated counterparts from the same tissue of origin. Similarly, in many cancer types, there is experimental proof that cellular dedifferentiation correlates with increased metastatic ability and poor patient prognosis (10-12). In papillary thyroid tumors, the loss of follicular cell differentiation is accompanied by disrupted NaI symporter (NIS) function and compromised iodide uptake avidity, resulting in RAI treatment resistance. Thus, patients with aggressive PTC variants often require multiple doses of radioactive iodine or repeated surgeries to address the metastatic disease. Although RAI resistance is associated with unfavorable prognosis like disease recurrence and even death (13-18), the dedifferentiation-inducing mechanisms in PTC cells still remains unclear.

One known mechanism for dedifferentiation-induced cancer progression and metastasis is the loss of expression of lineage specific transcription factors (19-22).

These tissue-specific transcription factors induce cell-fate specifications and promote differentiation in normal tissues. Studies have shown that such transcription factors, required to maintain adult tissue differentiation have a suppressive role in cancer progression (19-22). Thyroid follicular cell differentiation is sustained by the co-existence of the transcription factors, TTF1 and Pax8, and the regulation of these transcription factors in PTC is unclear (23). Given the involvement of PDGFRα in recruiting PTC cells into the EMT program for the acquisition pro-metastatic phenotypes (Chapter 4), further assessment of its role in PTC cell dedifferentiation was performed. The biological processes responsible for PDGFRα-driven dedifferentiation in PTC were outlined, as judged by TTF1 and Pax8 expression as well as iodide uptake avidity.

5.2 Results

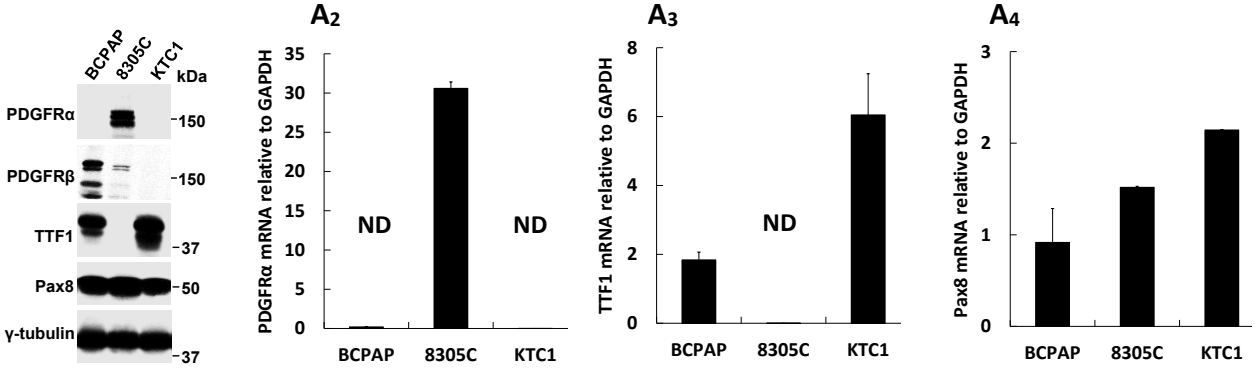
Expression of TTF1, but not Pax8, is Downregulated by PDGFRα in Dedifferentiated, Metastatic PTC

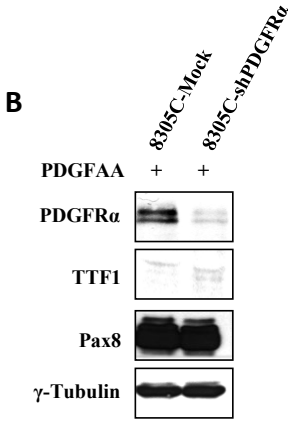
Co-expression of TTF1 and Pax8 is necessary for generating differentiated thyroid follicular cells that are capable of iodide transport (23-25). First, three papillary thyroid cancer cell lines were assessed for their differentiation status as judged by the presence of TTF1 and Pax8, and correlated with PDGFRα expression. A strong link between PDGFRα, loss of TTF1 expression, and dedifferentiation in these thyroid cancer cell lines was revealed (**Fig. 5.1A$_{1-4}$**). All the cancer cell lines expressed Pax8 protein, whereas TTF1 was present in only BCPAP and KTC1 cel1s which lacked PDGFRα expression (**Fig. 5.1A$_1$**). Conversely, the co-expression of PDGFRβ and TTF1 was observed, notably in the BCPAP cells. Pax8 expression was unaffected by the presence or absence of either PDGFRα or PDGFRβ. We confirmed the reciprocal expression of TTF1 and PDGFRα at the mRNA level through qPCR analysis (**Fig. 5.1$_{2-4}$**).

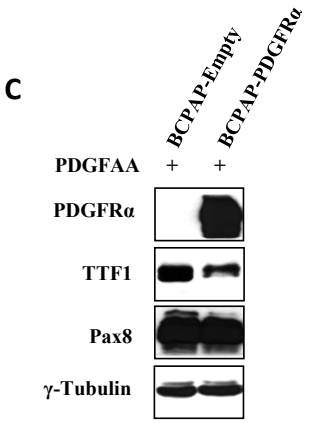
Next, the hypothesis that the presence PDGFRα disrupts TTF1 expression was tested using stably transfected PTC cell lines, BCPAP (native PDGFRβ), and 8305C (native PDGFRα). All thyroid cancer cell lines express significant levels of PDGF ligands BB providing for endogenous signal activation of the receptors (26). **Figs. 5.1B and 5.1C** show representative Western blots from the cell lines BCPAP and 8305C, respectively, in which we demonstrate that PDGFRα protein expression reduced TTF1 protein levels in BCPAP cells. Conversely, disruption of PDGFRα

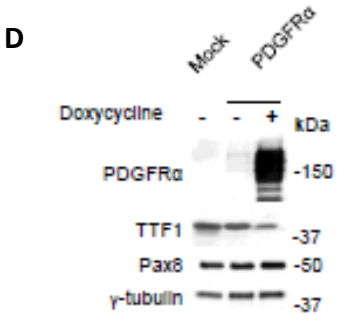
expression increases TTF1 protein levels in 8305C cells. It is noteworthy that the presence of native PDGFRβ in BCPAP cells (**Fig. 5.1A$_1$**) did not salvage TTF1 expression. Likewise, in primary cells derived from benign neoplastic thyroid tissue, forced expression of PDGFRα also decreased TTF1 expression significantly (**Fig. 5.1D**). No relationship seems to exist between Pax8 expression and the level of PDGFRα in primary cultures and PTC cell lines (**Figs. 5.1B-5.1D)**.

The association between PDGFRα, TTF1 and Pax8 expression was further investigated in clinical samples. Primary tumors lacking clinical evidence of metastases largely exhibited TTF1, but not PDGFRα expression. On the other hand, majority of the metastatic specimens expressed high levels of PDGFRα with minimal TTF1 staining (p=0.005; **Fig. 5.1E**). Pax8 protein expression was not influenced by the presence of the metastatic disease. So far, PDGFRα expression correlates with the metastatic disease, and it is inversely related of TTF1 protein levels in cell lines and clinical specimens. These results suggest that PDGFRα is an important contributor to follicular cell dedifferentiation during PTC metastases and progression.


BCPAP
8305C
KTC1
kDa
PDGFRα
150
PDGFRβ
150
TTF1
37
Pax8
50
γ-tubulin
37
A2
PDGFRα mRNA relative to GAPDH
ND
ND
BCPAP
8305C
KTC1
A3
TTF1 mRNA relative to GAPDH
ND
BCPAP
8305C
KTC1
A4
Pax8 mRNA relative to GAPDH
BCPAP
8305C
KTC1


B
8305C-Mock
8305C-shPDGFRα
PDGFAA + +
PDGFRα
TTF1
Pax8
γ-Tubulin


C
BCPAP-Empty
BCPAP-PDGFRα
PDGFAA + +
PDGFRα
TTF1
Pax8
γ-Tubulin


D
Mock
PDGFRα
Doxycycline - - +
kDa
PDGFRα
150
TTF1
37
Pax8
50
γ-tubulin
37

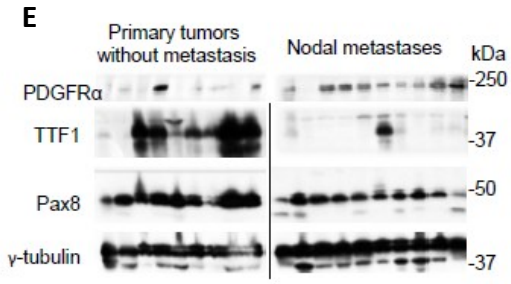

E
Primary tumors without metastasis
Nodal metastases
kDa
PDGFRα
250
TTF1
37
Pax8
50
γ-tubulin
37

Figure 5.1: PDGFRα expression induces dedifferentiation in thyroid cancer cells by disrupting TTF1 protein expression. (A) Western blot of thyroid cancer cell lines BCPAP, 8305C, and TPC1 demonstrate an inverse relationship between PDGFRα and TTF1 expression **(A_1)**. Comparative, quantitative PCR analysis of mRNA levels in papillary thyroid carcinoma cell lines confirming inverse relationship between PDGFRα **(A_2)** and TTF1 **(A_3)**. Pax8 mRNA expression is also shown **(A_4)**. Values relative to GAPDH; ND: Not detected. Data are presented as mean ± SEM. **(B)** Selective knockdown of PDGFRα in dedifferentiated thyroid cancer cell line 8305C restores TTF1 expression. **(C)** Western blot of BCPAP cells with inducible expression of PDGFRα under a doxycycline promoter disrupts TTF1 expression. **(D)** Western blot demonstrating that forced expression of PDGFRα in histologically normal thyroid tissue decreased TTF1 expression. Cells transduced with empty vector served as a control (mock). **(E)** In freshly prepared biological specimens, primary tumors without metastases typically lack PDGFRα, but metastases commonly express PDGFRα with subsequent loss of TTF1.

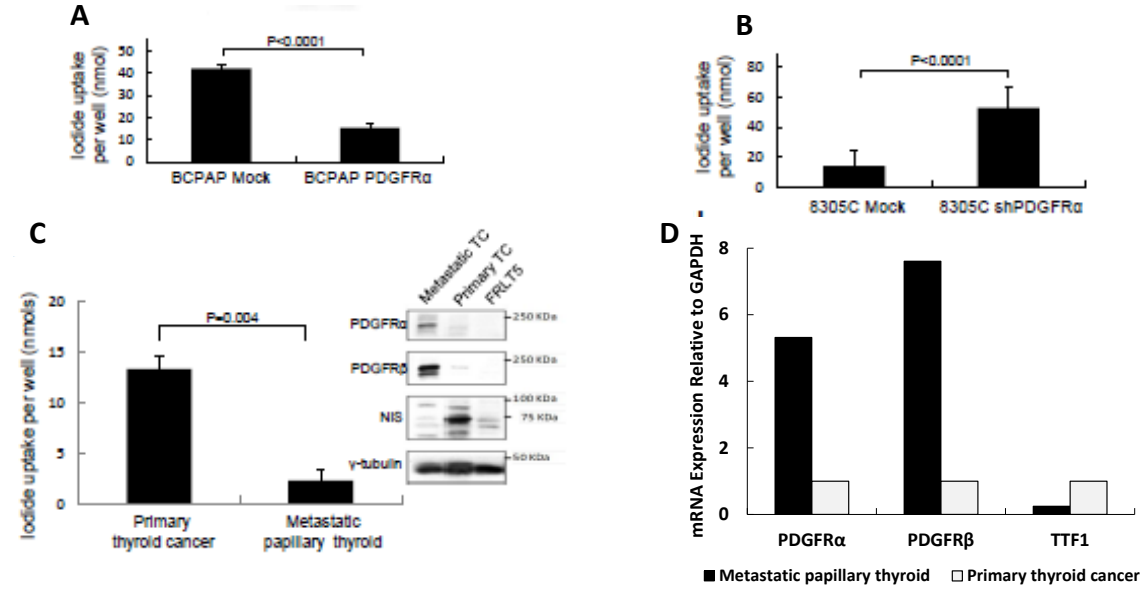
PDGFRα Expression Suppresses NIS Expression and Iodide Transport in Established and Primary PTC Cells

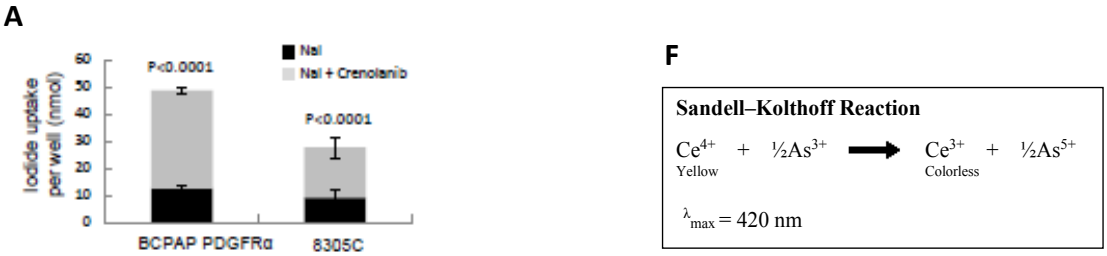
The surrogate measure of functional differentiation in thyroid cancer cell lines is usually NIS expression and ultimately iodide transport. As expected, forced expression of PDGFRα in BCPAP cells suppressed iodide transport (**Fig. 5.2A**), while the knockdown of PDGFRα in 8305C had the opposite effect (**Fig. 5.2B**). Resistance to RAI ablation is a prominent feature of disease recurrence and poor outcomes in PTC patients. Assessments of individual freshly isolated PTC primary cells also revealed that the NIS protein expression and transport function are significantly diminished in the presence of PDGFRα (**Fig. 5.2C**). Metastatic PTC cells expressing PDGFRα concentrated lesser amount of iodide, compared to the non-metastatic primary cells which barely expressed PDGFRα. A corresponding downregulation of the NIS protein due to the presence of PDGFRα, was observed in these primary thyroid cells (**Fig. 5.2C; inset**). Diminished differentiation was also evident as suppressed *TTF1* gene expression in the metastatic PTC primary cells harboring higher levels of the *PDGFRα* gene, compared to the non-metastatic cells (**Fig. 5.2D**). Pharmacologic blockade of PDGFRα signaling using Crenolanib restored iodide transport in BCPAP-PDGFRα and 8305C cells, thus providing a proof of principle for a role for PDGFRα-blockade in treating RAI resistant PTC patients **(Fig. 5.2E**).

Validation of Colorimetric Iodide Uptake Assay

The colorimetric iodide uptake assay used here is based on the Sandell–Kolthoff reaction (**Fig. 5.2F**). When cerium (IV) [Ce (IV)] and arsenic (III) [As (III)] solutions are successively added to the cell lysate, the reaction mixture changes from yellow to colorless in proportion to the iodide concentration. The reduction of yellow Ce(IV) to colorless Ce(III) by As(III) proceeds very slowly, and minute quantities of iodide can accelerate this reaction. The reaction is allowed to proceed for 60 min before the absorbance is read at 420 nm. The absorbance is then compared to a calibration curve generated under the same conditions with iodide standards (27).

Assay validation was performed by measuring the cell-trapped iodide in FRTL5 cells grown on 96-well plates. These are normal rat follicular thyroid cells which uptake iodide consistently (27, 28). **Fig. 5.2G** shows results from 16 uptake wells (with NaI only), 8 uptake inhibition wells (with both NaI and $NaClO_4$) and 8 background control wells (without NaI and $NaClO_4$). In the uptake wells, the average concentration of cell-trapped iodide was 132.7 ± 4.0 nmol. Replicate wells in the uptake inhibition and background control groups showed consistent signals, with values of 14.7 ± 0.66 nmol and 18.1 ± 0.32 nmol respectively.


A
Iodide uptake per well (nmol)
P<0.0001
BCPAP Mock
BCPAP PDGFRα
B
Iodide uptake per well (nmol)
P<0.0001
8305C Mock
8305C shPDGFRα
C
Iodide uptake per well (nmols)
P=0.004
Primary thyroid cancer
Metastatic papillary thyroid
Metastatic TC
Primary TC
FRLT5
PDGFRα
PDGFRβ
NIS
γ-tubulin
250 KDa
250 KDa
100 KDa
75 KDa
50 KDa
D
mRNA Expression Relative to GAPDH
PDGFRα
PDGFRβ
TTF1
Metastatic papillary thyroid
Primary thyroid cancer


A
Iodide uptake per well (nmol)
NaI
NaI + Crenolanib
P<0.0001
P<0.0001
BCPAP PDGFRα
8305C
F
Sandell–Kolthoff Reaction
Ce4+ + ½As3+ → Ce3+ + ½As5+
Yellow
Colorless
λmax = 420 nm

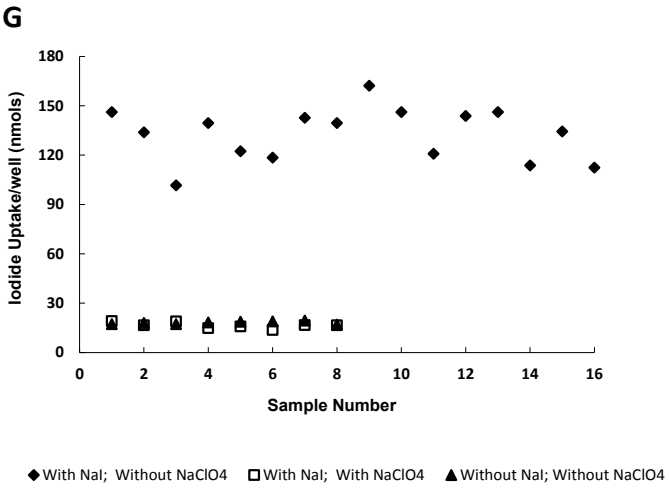

G
Iodide Uptake/well (nmols)
Sample Number
With NaI; Without NaClO4
With NaI; With NaClO4
Without NaI; Without NaClO4

Figure 5.2: PDGFRα protein expression culminates in the disruption of follicular cell function. The consequential of PDGFRα expression on sodium iodide transport in BCPAP cells **(A)** and 8305C **(B)** cells. **(C)** Primary thyroid cultures that were prepared from freshly isolated cells from patients show the inverse relation between iodide transport and PGFRα expression in primary thyroid cancer versus metastatic PTC. Results are means ± SEM, n=6. **Inset:** Western blots of corresponding primary thyroid cultures revealing predicted changes in sodium iodide symporter (NIS) protein levels due to expression of PDGFRα, rat FRTL5 thyroid cells are shown for comparison. **(D)** Gene expression levels of PDGFRα, PDGFRβ, and TTF1 in the primary thyroid cultures. Results are expressed as mean of duplicate experiments. **(E)** Effect of Crenolanib treatment on sodium iodide transport in PDGFRα-expressing BCPAP and 8305C cells. Results are means ± SEM, n=8 of three independent experiments. **(F)** The Sandell–Kolthoff reaction between As (III) and Ce (IV), catalyzed by iodide. **(G)** Iodide uptake assay performance assessed in FRTL5 cells. Assay was performed as described in the methods section, with 16 uptake wells (NaI only), 8 uptake inhibition wells (both NaI and $NaClO_4$) and 8 background control wells (without NaI and $NaClO_4$).

PDGFRα and Cytoplasmic TTF1 Levels are Prognostic Markers of Recurrence, and Radioactive Iodide Resistance in PTC

This relationship between PDGFRα and TTF1 levels was further explored using tissue arrays which included 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. As described in previous reports, the cytoplasmic staining of PDGFRα and PDGFRα was assessed for each case, in triplicate, as 3+, (strong, diffuse), 2+ (strong, focal), 1+ (weak staining), or 0 (minimal staining) (26).

Clinical data was obtained from a prospective database maintained at the Cross Cancer Institute tracking disease from 2002 onwards. 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 were obtained directly from the radioisotope records maintained at the Cross Cancer Institute. Clinical follow-up data for an average of 7.9 years (range 2.4 years to 11.1 years) for the donors was accessible. For each histological group, patient age, sex, tumor size and lymph node status are outlined in **Table 5.1**.

Immunohistochemical staining of PDGFRα in tumor samples correlated strongly with the nucleus-cytoplasmic shift in TTF1 staining, and this was especially pronounced in the metastatic disease. In contrast, node negative PTC specimens

exhibited more nuclear staining than cytoplasmic. Histologically normal or benign lesions consistently showed strong and specific nuclear staining for TTF1 (**Fig. 5.3A**). As reported in Chapter 1, PDGFRα was found at much higher levels in PTC specimens with nodal metastases compared to primary tumors lacking evidence of metastases (**Fig. 5.3A**). It is noteworthy that Pax8 staining was predominantly nuclear in all histological groups. (**Fig. 5.3A**). The scoring summary for the immunohistochemistry is shown in **Table 5.2** and the quantification of the mean nuclear staining intensities of TTF1 and PDGFRα as a function of tissue type is shown in **Fig. 5.3B**. PTC tumor specimens with high PDGFRα staining were over three times more likely to exhibit nodal metastases ($P<0.0001$), and were larger ($P=0.03$) than those with minimum PDGFRα staining. An inverse association was observed with TTF1 staining, where high nuclear staining levels correlated with smaller tumor size ($P=0.0001$) and absence of nodal metastases ($P=0.0008$). Dosimetry data reveals that patients whose tumors exhibited higher PDGFRα ($P=0.005$) or lower nuclear TTF1 ($P=0.007$) staining received significantly higher therapeutic doses of RAI (**Fig. 5.3C**).

Table 5.1: Tissue array distribution

Tissue	Age (mean)	Female (male)	Tumor size (cm)	Nodes assessed (mean)
Normal (n=36)	47.1 ±15.8	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

Table 5.2: 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

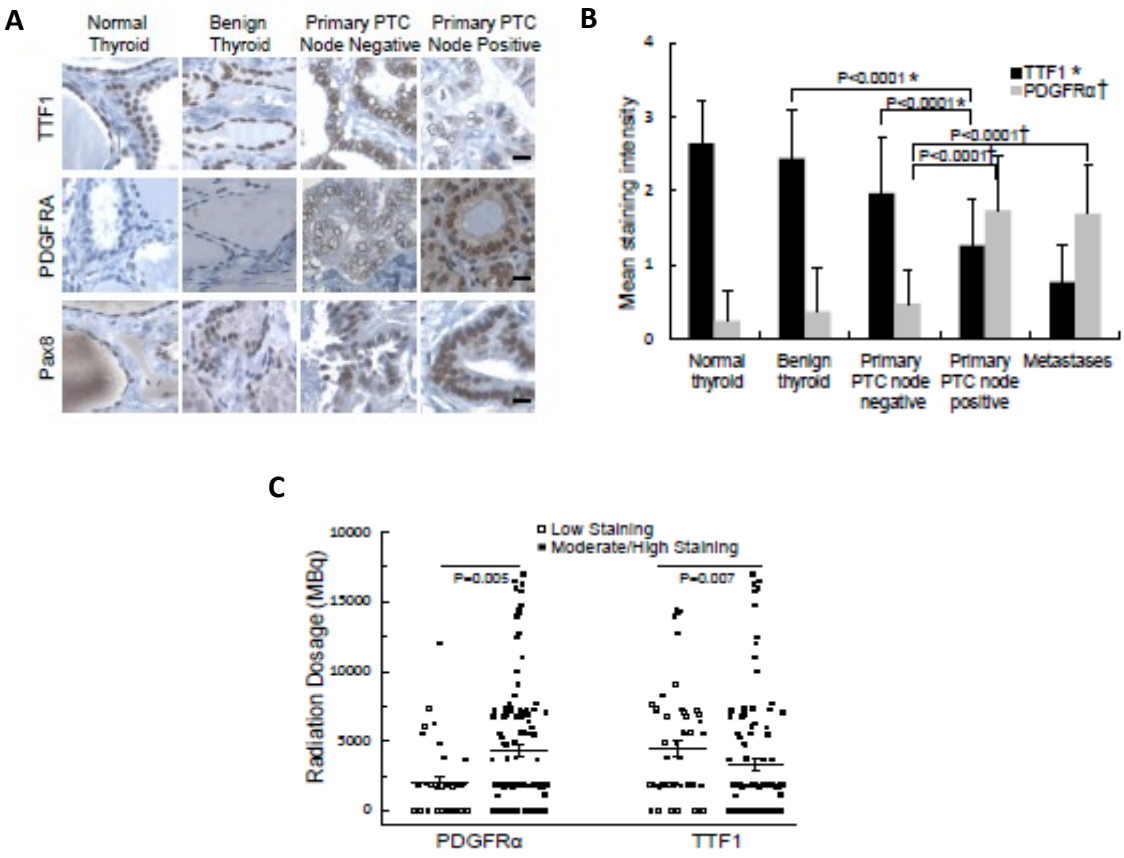
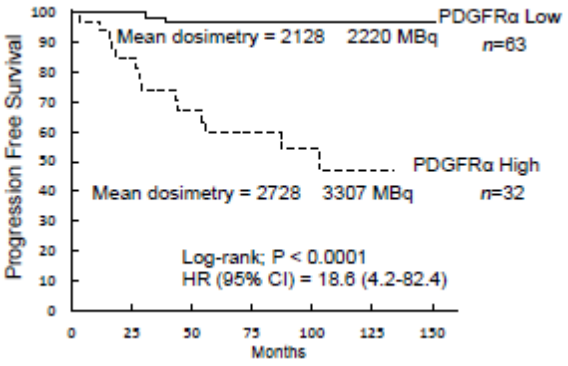


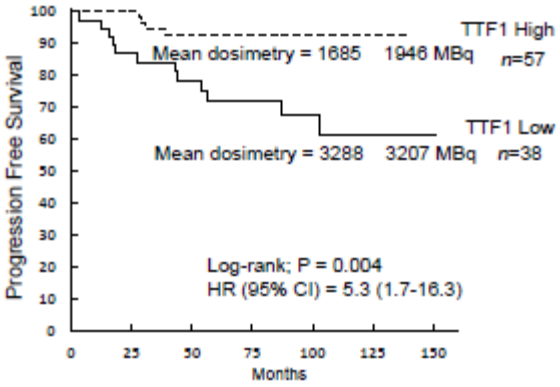
Figure 5.3: High PDGFRα expression in human metastatic PTC disrupts nuclear TTF1 expression, and is prognostic of disease recurrence and RAI resistance. **(A)** Representative immunohistochemical staining for TTF1 (top), PDGFRα (middle) and Pax8 (bottom) in normal thyroid, benign neoplasms and primary tumors with, and without, nodal metastases. Scale bars 50 μm. **(B)** Mean staining intensities for TTF1 and PDGFRα proteins in arrayed thyroid clinical specimens including normal tissue, benign neoplasms, primary PTC and metastatic PTC. Results are means ± standard errors, N as described in **Table 5.1**. **(C)** Scatter plot showing dosimetry distribution in patients as a function of PDGFRα and TTF1 tumour staining by immunohistochemistry

We also examined a cohort of patients with initial absence of ultrasound or biochemical (thyroglobulin (TG) <0.4ng/ml) evidence of nodal metastases, which were routinely monitored. Patients were assessed for disease recurrence as defined by positive ultrasound scans, unstimulated TG level (>0.4 ng/ml) or stimulated Tg level (>2.0 ng/ml). PDGFRα-positive tumors were more than 18 times more likely to recur (95% CI: 4.2-82.4, P<0.0001) than those lacking PDGFRα even when matched for radiation dosimetry (P=0.28) (**Fig. 5.4A)**. Low levels of TTF1 nuclear expression correlated strongly with recurrent disease (HR=5.3 95% CI: 1.7-16.3, p=0.004) even when they were subjected to higher doses of radiation (P=0.003) (**Fig. 5.4B**). These results were consistent for patients above or below 45 years of age as shown in **Figs. 5.4C and 5.4D**. Given the inverse relationship between TTF1 and PDGFRα, the overall expression of TTF1 and the nuclear/cytoplasmic ratio may be very useful surrogates to identify the risk of RAI resistance and a potential for treatment with PDGFRα blockade. In line with our hypothesis, PDGFRα seems to be an essential pathway to target in the treatment of aggressive, RAI resistant PTC variants which have metastasized to the lymph nodes.

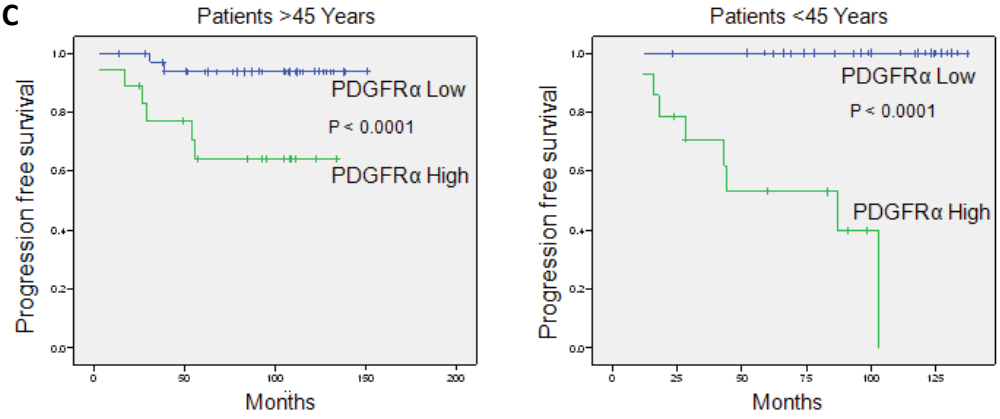
A


Progression Free Survival
PDGFRα Low
Mean dosimetry = 2128 2220 MBq
n=63
PDGFRα High
Mean dosimetry = 2728 3307 MBq
n=32
Log-rank; P < 0.0001
HR (95% CI) = 18.6 (4.2-82.4)
Months

B


Progression Free Survival
TTF1 High
Mean dosimetry = 1685 1946 MBq
n=57
TTF1 Low
Mean dosimetry = 3288 3207 MBq
n=38
Log-rank; P = 0.004
HR (95% CI) = 5.3 (1.7-16.3)
Months

C


Patients >45 Years
Progression free survival
PDGFRα Low
P < 0.0001
PDGFRα High
Months
Patients <45 Years
Progression free survival
PDGFRα Low
P < 0.0001
PDGFRα High
Months

D

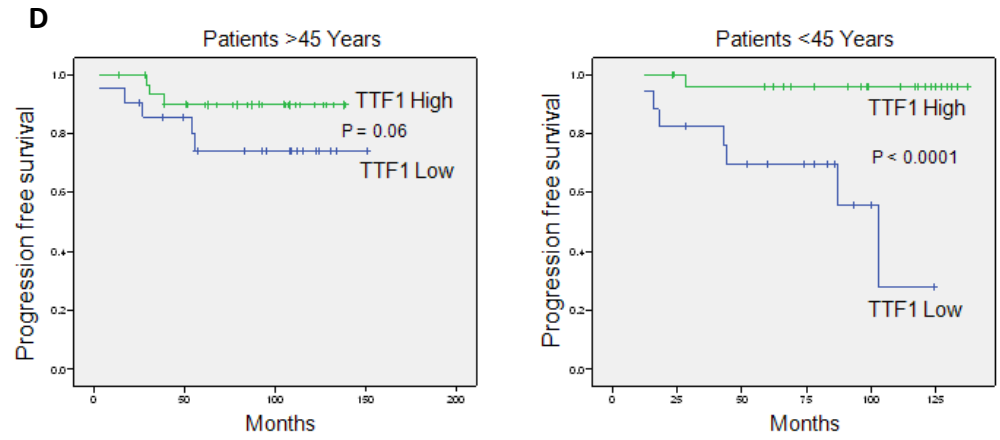

Patients >45 Years
Progression free survival
TTF1 High
P = 0.06
TTF1 Low
Months
Patients <45 Years
Progression free survival
TTF1 High
P < 0.0001
TTF1 Low
Months

Fig. 5.4: Kaplan-Meier plots of disease recurrence as a function of time based on PDGFRα **(A)** and TTF1 **(B)** immunohistochemical staining. 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 **(C)** and TTF1 **(D)** immuno-histochemical staining.

5.3 Discussion

In this current body of work, for the first time a down-regulatory mechanism for the differentiation factor, TTF1, was identified for papillary thyroid cancer. Previously, the machinery that triggers PTC cell dedifferentiation and consequent resistance to RAI therapy was undefined, particularly in the metastatic disease. Dedifferentiation to a higher histologic grade is associated with increased aggressiveness in many cancers (29-32). Following the demonstration that differentiated mammary epithelial cells could be reprogramed into less differentiated epithelial stem cells with mesenchymal traits. In fact, the EMT is now considered to be a dedifferentiation program associated with cancer metastasis, in which epithelial cell–cell junctions are disrupted (33-35). E-cadherin is one of the junctional protein, whose downregulation is an important indicator of cells undergoing EMT. A dedifferentiated phenotype with decreased E-cadherin expression has been correlated with nodal or distant metastasis in prostate, head and neck, breast, colorectal, gastric, and lung cancers (36-41). We further proposed that PDGFRα drives PTC cell dedifferentiation given that PDGFRα was implicated in the recruitment of PTC cells into the EMT program as they acquired pro-metastatic phenotypes (Chapter 4).

The requirement of TTF1 and Pax8 in thyroid follicular cell function has been well defined and demonstrated by Antonica and colleagues (23). Following embryogenesis, the secretion of thyroid hormone by the thyroid gland requires iodide transport, which is always dependent on TTF1 and Pax8 activity. The concept of disrupted differentiation and cancer progression has been found in other tissues. The

loss of transcription factors required for the maintenance of adult tissue differentiation is a known initiator of dedifferentiation - mediated cancer progression and metastasis (19-22). For instance, reduced Elf5 expression led to the induction of EMT and metastasis in mouse models of breast cancer (22). Normally, Elf5 promotes breast alveolar morphogenesis and regulates mammary stem cells function (22). In the same manner, the loss of Nkx3, a regulator of prostate epithelium differentiation, correlates with tumor progression in prostate cancer (19). It is clear in the literature that TTF1 and Pax8 are both important transcriptional regulators of thyroid follicular cell differentiation and function (23, 42). Thus, in an attempt to determine if PDGFRα has a direct role in dedifferentiation, its expression was assessed as a function of TTF1 and Pax8 levels in PTC cells and clinical specimens. The influence of PDGFRα on functional differentiation was also assessed, as judged by iodide transport in primary and established PTC cells.

First, we found that PDGFRα has the unique ability to mediate thyroid follicular cell dedifferentiation by downregulating TTF1 expression. In clinical specimens, we observed that TTF1 was increasingly lost and localized to the cytoplasm in metastatic tumors, which exhibited stronger PDGFRα staining. Similarly, in PTC cells, TTF1 levels diminished in the presence of PDGFRα, resulting in compromised sodium iodide transport. The cell lines used harbor different mutations, including BRAF V600E (BCPAP, KTC1), and p53 (8305C - a poorly differentiated form of PTC). Thus the inverse relationship between PDGFRα and TTF1 expression is not a reflection of a single cell line variant or activation of a

single pathway (e.g. ERK). Pax8 expression levels and localization were not impacted by PDGFRα in PTC cells and clinical samples.

Resistance to RAI therapy is a central feature of disease recurrence and poor outcomes in thyroid cancer. Regarding clinical outcomes, patients exhibiting PDGFRα were three times more likely to have nodal metastases and they required nearly twice the dose of RAI, compared to those lacking PDGFRα expression in their tumors. Likewise, patients lacking clinical evidence of nodal metastasis at diagnosis, but with PDGFRα-positive tumors, were much more likely to recur with the disease compared to patients lacking PDGFRα, even when matched for patient age. Differences in survival were not observed in this cohort, but clear-cut evidence from previous studies show that recurrent PTC is predictive of increased mortality (43). It is also well-documented that increased use of radioactive iodine and repeated surgeries increase morbidity including lifelong hypoparathyroidism and sialoadenitis (44). Thus, it is clear that the current modalities of therapy have significant disadvantages to patients that could be remedied by targeted therapy in the form of PDGFRα blockade.

We have also provided a clinically valuable demonstration that specific inhibition of PDGFRα activation in PTC cells will restore iodide uptake avidity. Canonically speaking, PTC is largely MAPK/Erk-driven (especially via *BRAF* gene mutation), but subsets of tumors with varied differentiation levels exist within this broad classification (45). Treatments targeting the MAPK/Erk pathway including resveratrol, rapamycin, retinoic acid, and multiple tyrosine kinase receptor inhibitors

have all been assessed for their redifferentiating and tumor-rescinding abilities (46-57). Outcomes have been conflicting and there is no clinical evidence suggesting that this pathway affects differentiation and is an effective target for aggressive variants of PTC. As such, TTF1 and Pax8 regulation in papillary thyroid tumors could be independent of the MAPK/Erk pathway (43). In the absence of a targeted therapy for dedifferentiated metastatic PTC, empirically-driven clinical trials of different tyrosine kinase inhibitors that disrupt VEGFR, FGFR and other signaling pathways have been used. Besides the widely varied response rates that were transient and accompanied with significant toxicity, drug-related deaths were also recorded (58, 59). As yet, treating the advanced disease is a challenge to clinicians (54). However, this results presented in this Chapter are a proof of principle that selective blockade of PDGFRα signaling could significantly improve radioactive iodide treatment and decrease metastases in PTC.

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CHAPTER SIX: CONCLUSIONS, FUTURE DIRECTIONS AND TRANSLATIONAL IMPLICATIONS

6.1 PDGFRα as a diagnostic test and a therapeutic target in papillary thyroid cancer

We have demonstrated that PDGFRα is a driving force behind the metastatic disease in papillary thyroid cancer, which accounts for nearly 30,000 deaths per year worldwide (1). The presence of this tyrosine kinase receptor in recurrent, metastatic forms of papillary thyroid cancer (PTC) makes it an important target and a new option for diagnosing and possibly treating cases resistant to the present therapy regime, which combines surgical resection with RAI ablation of remnant thyroid and metastatic deposits. The increased levels of PDGF-AA, which favor PDGFRα activation, represents a possible diagnostic toolkit that could be identified as a risk for tumor recurrence, and this could be used to warn patients and physicians of the need for additional therapy. The benefit is that the threats associated with increased therapy surgically or with radioactive iodine (including lifelong thyroid hormone-replacement therapy, recurrent laryngeal nerve damage) would be limited to those patients in the highest risk category (2). This would reduce the significant psychoeconomic consequences, which abound in patients with recurrent PTC. The use of PDGFRα and/or its corresponding PDGF-AA ligand could also guide subsequent ultrasound surveillance, serologic and radiographic testing, and accumulating health care costs as part of the lifelong monitoring for disease persistence/recurrence (3, 4).

From a surgical perspective, the link between PDGFRα and lymph node metastasis could also be a tool to guide the extent of surgery. Current disease management strategies cannot predict metastatic risk, and the extent of the initial surgery is usually left to the physician's discretion (4). This quandary, caused by compromised ultrasonographic assessment of lymph node status in the central neck compartment (because of the overlying thyroid gland), severely restricts the ability to make informed decisions on the appropriate extent of surgery (3, 5-9). With intra-operative examination, even with experienced surgeons, the sensitivity and specificity of identifying metastasized lymph nodes can be poor (3). Therefore, very often, it becomes a significant challenge to decide who should receive lymph node resection, especially when it is prophylactic. This challenge is even reflected internationally as discordance among surgical guidelines (10, 11). For instance, in the United States, total or partial thyroidectomy without prophylactic node resection is endorsed (10), while the Japanese guidelines suggest prophylactic central neck lymph node resection (+/− lateral neck lymph node dissection) at the time of primary surgery due to high incidence of microscopic lymph node metastases with PTC (11). The lack of any other test linked to nodal metastases, which is virtually absent in normal or benign tissue, makes PDGFRα a good candidate for further study in a prospective clinical setting.

Another peril associated with the nodal spread in PTC patients is tumor resistance to RAI therapy. We believe that we have defined the first mechanism that regulates the differentiation of follicular cells in papillary thyroid cancer through the

down regulation of TTF1 by PDGFRα. A patient is defined as having RAI-refractory disease if there is at least one lesion without RAI uptake or if a lesion has progressed within a year following RAI treatment or persisted after the administration of a cumulative activity of more than 600 mCi. Thirty% of metastatic differentiated thyroid cancer (of which the majority are PTCs) become refractory to RAI ablation, and higher (or repeated) doses are often administered in these cases (14). However, evidence suggesting that RAI is of little benefit in patients with RAI-refractory disease exists (12, 13). It is under this circumstances that PDGFRα blockade has potential utility as a drug target in the treatment of metastatic PTC tumors, particularly those resistant to RAI ablation. Combining the use of a tyrosine kinase receptor inhibitor that blocks PDGFRα signaling, could avoid the need for repeated dosage of RAI, which is associated with complications like salivary gland damage, dental cavities, nasolacrimal duct obstruction, and secondary malignancy (14, 15). Thus overall we propose that this body of work has very clearly demonstrated a central role of PDGFRα in papillary thyroid cancer tumorigenesis that has the potential to generate new diagnostic and therapeutic tools for better patient care.

6.2 A novel diagnostic test encompassing PDGFRα in PTC

While there are many studies that examine the utility of genotyping and Microarray assessment of fine needle aspiration biopsy's predicting malignancy in the context of indeterminate lesions, there has been very little work on predicting

metastatic behavior in known or expected cancers. Several studies have shown that the expression of the BRAF (V600E) oncogene is positively associated with lymph node metastasis in PTC and it is frequently used to predict the probability of nodal spread (16). However, the uncertainty regarding the predictive power of BRAF (V600E) has been reported (17), and a recent revelation that at least four different molecular clusters with variable degrees of thyroid differentiation exist within the BRAF (V600E) positive tumors could explain this (18). These authors performed a comprehensive genomic analysis of 496 PTCs (the largest cohort studied to date), and their results further recommend a reclassification of papillary thyroid tumors into molecular subtypes that better reflect their underlying signaling properties (18). However despite all of this effort, a singular test predicting metastases has remained elusive. We show that the expression of PDGF-AA and PDGFRα is significantly more pronounced in PTC specimens with lymph node metastases, suggesting that they could be prognostic of metastatic risk. Other human cancers reported to express PDGFRα with prognostic implications include renal, breast, ovarian, prostate, melanoma, and bone cancer (19-24). Similar to our finding, malignancy-linked co-expression of PDGF and PDGF receptors have been observed in sarcomas (25-28). Increased expressions of PDGF ligands and receptors were observed in glioma cell lines (29, 30) and in patient samples (31-37). PDGF-DD and PDGFRβ were found to be co-expressed in several mesothelioma cell lines, resulting in autocrine stimulation of cell proliferation (38). These studies provide further substantial evidence that

PDGFRα signalling could be apparent in differentiated tissues driving malignancy and thus a marker worthy of further investigation.

In our retrospective cohort with immunohistochemistry, PDGFRα was used to predict the presence of metastasis in surgically-derived tumor specimens. The sensitivity and specificity that were achieved were 86% and 71%, respectively. These clinically relevant values suggest the potential utility of PDGFRα staining in helping to decide the extent of surgical intervention, especially prophylactic lymph node dissection. However, there are technical challenges. The current study used immunohistochemistry, which of course means that the tumor specimens have been already resected and could not drive surgical decision-making. To be of most value, we would need to consider tests in the form of a platform that supports analysis of fine needle aspirates. Fine needle aspiration biopsy (FNAB) is currently the recommended preoperative specimen for the clinical diagnosis of solid thyroid nodules larger than 1 cm (39-41). For preoperative detection, ELISA or qPCR analyses are appealing alternatives to IHC, as they can be performed on small amounts of biopsy material. ELISA or qPCR analyses have been used successfully to detect diagnostic and prognostic biomarkers in fine needle aspiration biopsies (FNAB) of breast cancer and melanoma patients (39, 40, 42, 43). The detection of differential PDGF-AA concentrations in serum of PTC patients by ELISA could also serve as a non-invasive testing platform. As it stands, the future studies that we would propose would likely entail a matched prospective cohort where fine needle aspirates that were assayed for the presence of mRNA for PDGF-AA cytokine or PDGFRα are

matched to the subsequent final pathology for patients with and without lymph node metastases.

Compartment-based removal of neck lymph nodes in appropriate patients is effective in preventing the recurrence of PTC (3). Knowing this, in consideration of future clinical cohort testing, using PDGFRα as a surrogate of metastases for preoperative surgical decision making, the goal would be to reduce recurrence rate significantly. For instance, with a strong preoperative PDGFRα staining, prophylactic lymph node dissection in the absence of apparent pathologic lymph nodes could be appropriate. According to our findings, there is high probability that PDGFRα-driven nodal metastases of PTC will be insensitive to RAI ablation due to impaired radioiodine avidity. Hence, the risk of recurrence will increase if microscopic nodal metastases were present, and the lymph nodes were not removed. Additionally, this PDGFRα staining-assisted preoperative prognosis could be specifically valuable for the management of usually low-risk PTC (such as PTC with low TNM staging and papillary thyroid microcarcinoma). It has been reported that this group of obviously low-risk patients can also progress with recurrence, though at a lower rate than conventionally high-risk patients (3). Thus, preoperative PDGFRα testing could help to identify the more aggressive ones for somewhat more comprehensive initial surgical treatments. At the same time, with this approach, the majority of the low-risk patients would be spared from unnecessary prophylactic lymph node dissection. Consequently, surgical complications are likely to reduce significantly since fewer patients would need such high-risk surgeries. Indeed, we have shown with compelling

evidence that PDGFRα staining of biopsies can be a novel and useful prognostic strategy to assist preoperative metastatic risk stratification and tailored surgical management of PTC patients.

6.3 PDGFRα up regulates multiple signaling pathways beyond the canonical description of the MAPK/Erk-associated carcinogenesis in papillary thyroid carcinoma

The current work redefines the traditional canonical description of papillary thyroid carcinoma as a MAPK/ERK driven cancer. This traditional description is challenged by a clear demonstration of a vital role for the STAT3 and AKT pathways in driving aggressive disease phenotype in PTC cells. STAT3 signaling was strongly implicated in effecting the pro-metastatic signals from PDGFRα, while the PI3K/Akt and MAPK/ERK pathways seem to play lesser or adjunct roles. According to current knowledge, activation of the MAPK/Erk pathway by genetic alterations (like BRAFV600E and RET/PTC mutations) primarily drives the development of PTC. By contrast, many of the genetic alterations that activate the PI3K/Akt pathway are generally less common in PTC, so this pathway plays a lesser role in the transformation of thyrocytes to this histological variant of thyroid cancer (44). However, activation of the PI3K/Akt pathway in concurrence with the MAPK/ERK pathway has been implicated in the progression of PTC, from low-grade to aggressive variants (44).

Evidence from genetic data sets (45, 46) and the hyperphosphorylation of Akt observed particularly at the invasive tumor fronts (47), strongly echo a role for aberrantly activated PI3K/Akt pathway in driving aggressiveness in progressive PTC. Dual activations of these pathways are commonly seen in aggressive, dedifferentiated thyroid cancers, like recurrent/metastatic and anaplastic variants (44). Interestingly, this observation also seems to be applicable to other human cancers, like melanoma in which simultaneous activation of the PI3K/Akt pathway (via PTEN mutation) and the MAPK/Erk pathway (via BRAF mutation) led to aggressive progression and metastasis of the tumor (48). Given these previous findings, its capacity for the dual activation of the PI3K/Akt and MAPK/Erk pathways, further supports the role of PDGFRα as a driver of aggressiveness in papillary thyroid tumors. However, this is the first report identifying PDGFRα, as the initiator of this combined activation in PTC cells.

STAT3, which is a transcriptional factor known to be activated by numerous cytokines, growth factors, and oncogenic proteins, is constitutively phosphorylated in human cancer specimens and cell lines (49). Interestingly, these results corroborate a previous report (from our laboratory) in which histological analysis revealed an elevated expression of activated STAT3 in the nodal deposits of metastasis PTCs (50). However, the existing literature is void of reports on PDGFRα-induced activation of STAT3 in PTC cells. There is substantial evidence that STAT3 is involved in various stages of metastasis including tumor cell migration and invasion (51). Stat3 has been shown to transcriptionally activate the expression of genes that

promote tumor cell migration and invasion. Specifically, activated STAT3 regulates tumor invasion of melanoma cells by regulating the transcription of matrix metalloproteinase 2 (MMP-2) (52). By induction of MMP-1 and MMP-10, Stat3 activation promoted the migration and invasion of bladder cancer cells (53). It was also required for the MMP-9 production in transformed human breast epithelial cells (54).

Stat3 can also contribute to cancer cell migration and invasion through transcription-independent pathways. For instance, Stat3 has been shown to interact directly with the cell motility machinery such as microtubules and focal adhesions (55). In ovarian cancer cells, Stat3 protein was localized to focal adhesions and its interaction with phosphorylated focal adhesion kinase and paxillin enhanced the invasiveness of the cells. The involvement of multiple signaling pathways in aggressive PTC suggests that it may be necessary to target them simultaneously for effective treatment. Nonetheless, it is worthy of mentioning that PDGF-induced activation of STAT3, which led to the mitogenic transformation of fibroblast cells, has been documented (56).

From a clinical perspective the involvement of multiple pathways demonstrates the problematic approach of therapeutic agents that disrupt single pathway such as BRAF inhibitors. Compensatory responses to the blockade of a single pathway could be seen in our study. Therefore, future experiments assessing the effect of combined (and paired) inhibition of the STAT3, PI3K/Akt and MAPK/Erk pathways on surrogate markers of invasiveness such as the formation of

invadopodia, will be performed. The first dimension of further testing would include specific blockade of the individual pathways, looking for responses in SCID mice models following their inoculation with differentiated and undifferentiated PTC cells as used in this thesis. Ultimately a second dimension would involve the assessment of an array of upstream TKIs for their relative abilities to disrupt the different pathways. It is suspected that the toxicities of combining TKIs would be prohibitive in most circumstances. However, combining certain TKIs with focal blockade of STAT3, PI3K/Akt, or MAPK/Erk pathways may be favourable with minimal side effect profile.

6.4 The PDGFRα-induced mechanisms underlying the propensity of papillary thyroid cancer for lymph node metastases

We report for the first time in PTC cells that PDGFRα promotes invadopodia formation by sensitizing cells to EMT via Slug and Snail induction. Overexpression of PDGFRα in PTC cells culminated in augmented matrix degrading and migratory abilities, as Snail and Slug protein levels increased. Disruption of PDGFRα signaling resulted in significant downregulation of Snail and Slug expression as well as compromised migratory and gelatinase activity in PTC cells. Similar to this finding, Hardy *et al.,* (57) showed that Snail and Slug were highly expressed in cell lines derived from human PTC samples and their metastases, but no link with PDGFRα signaling was made. However, the involvement of PDGFRα in invadopodia

development has been demonstrated in breast cancer cells and tumor metastasis *in vivo* (58). A crucial role for PDGF-AA/PDGFRα signaling was also identified in hepatocytes undergoing TGF-β-induced EMT and tumor progression (59). Up-regulation of Snail and Slug with corresponding increases in aggressiveness as well as poor prognosis has been reported in many human malignancies (60-68). The ability of PTC cells to extend beyond the thyroid capsule into the surrounding neck tissue and metastasize to regional lymph nodes is critically dependent on the generation of motile cellular structures that can invade and degrade the capsular tissue and extracellular matrix.

Downregulation of the E-cadherin gene in PDGFRα-expressing cells further strengthens our proposal that PDGFRα recruits PTC cells into the EMT process. Snail and Slug are well-known repressors of E-cadherin and they also downregulate some components of the adherens junction to mediate cell-cell adhesion (69). E-cadherin is the major component of the epithelial adherens junctions, which along with tight junctions, mediate cell-cell adhesion (70, 71). Loss/downregulation of E-cadherin is a characteristic feature of cells undergoing EMT, which causes non-motile, epithelial cells to shed their intracellular adhesion molecules to become individual, motile mesenchymal-like cells. This process is believed to be crucial for invadopodia biogenesis, and consequent acquisition of migratory and ECM degrading abilities usually seen in metastasizing cancer cells (70, 71). Our results strongly suggest that invadopodia-mediated matrix degradation is a key function of PDGFRα in promoting PTC tumor metastasis.

Upregulation of the *SPP1* and *LPAR4* genes, which have been previously linked to invadopodia formation in cancer cells, was also seen in PDGFRα-expressing PTC cells. Elevated expression of SPP1 is strongly associated with enhanced aggressiveness, metastasis and poor prognosis in many cancers (72-75). Serum levels of SPP1 were found to be about 4- to 10-fold higher in a variety of disseminated human carcinomas, including breast, lung, and prostate (76); and these higher levels were correlated with higher tumor grade. In PTC patients, evidence of a positive association between the cytoplasmic overexpression of SPP1 and poor survival outcome exist (74). However, there is no evidence linking PDGF signaling to SPP1 expression/activity in any tumor type, this relationship is open for further validation in PTC. ATX/LPA signaling is positively associated with invasiveness and metastasis in several cancers including melanoma, breast cancer, ovarian cancer, thyroid cancer, renal cell cancer, lung cancer, neuroblastoma, glioblastoma multiforme, and hepatocellular carcinoma (77). Interestingly, a link between LPA signaling and SPP1 expression has been identified in gastric and hepatic cancer cells (78, 79).

To translate to clinical use, it will be reasonable to identify other surrogate makers of invasion that work with PDGFRα. In cancer cells, it is believed that aggressiveness is driven by a migratory phenotype that shares common pathways with inflammation (80). Our gene array results emphasize this interplay between migratory (PDGFRα-induced) and inflammatory (ATX/LPA-induced) signals during PTC metastasis. The mechanistic interactions between PDGFRα and ATX/LPA signaling, as well as their convergent roles in the nodal spread of PTC, warrant

further exploration. This is a very attractive project that will be prioritized in future studies, given that ATX and LPAR antagonists are already in clinical trials (80). Inhibiting LPAR4 activity could provide an effective and novel strategy for decreasing the inflammatory phenotype in PTC, and this could complement PDGFRα-specific TKIs. Clearly, more work still remains to be done to verify and expand this preliminary gene array result. Foremost, especially for the genes of interest, the differential expression observed still requires verification at the protein level. Furthermore, the understanding of their roles in the context of PDGFRα-mediated acquisition of aggressive features will require modulating them. This could be accomplished either through shRNA-mediated knockdown of those genes that are highly expressed, or overexpression of genes with low basal levels. Small molecule inhibition could also be a useful tool for this future exploration.

While we have provided evidence showing that invadopodia-mediated matrix degradation is an important part of the process through which PDGFRα promotes nodal metastasis in PTC, future work is needed. Observations that focal adhesions are also capable of degrading the ECM (81) have been made. Some investigators reported that invadopodia can be distinguished from focal adhesions by a lack of focal adhesion kinase (FAK) staining with immunofluorescence (82). In contrast, others have reported an essential role for FAK in invadopodia biogenesis (83). The notion that invadopodia can be formed through multiple mechanisms, with some arising from nascent focal adhesions and others from de novo actin polymerization (84, 85), has also been considered. These conflicting concepts raise the need for further

characterization of the invadopodial protrusions seen in PDGFRα expressing PTC cells.

It is also worth mentioning that the presumed role of invadopodia in cancer metastasis has been defined mostly by *in vitro* approaches, and the lack of substantial *in vivo* evidence still raises questions regarding their physiological relevance in cancer (86). The metastatic process is a complex one, requiring interactions of cancer cells with multiple host tissues and cell types, in vitro assays like the ones used in this study, lack the full array of these physiological interactions that take place in vivo (86). Given that metastases also occur over time and within organs generally hidden from direct observation, this process can be difficult to explore effectively. Though animal models of metastasis provide the physiological context, they still suffer from the inherent covert nature of the metastatic process (87). The chick embryo metastasis model lacks these highlighted limitations, and it has been successfully used in combination with intravital imaging tools to visualize the different aspects of metastasis (including invadopodia formation) (86, 87). The accessibility of the chorioallantoic membrane, a well-vascularised, thin tissue available on the outside of the embryo, makes this in vivo assay suited for microscopic observations and analyses (87). We seek to employ the chick embryo model for further exploration of the role of PDGFRα in PTC metastasis. It has been used to outline the dynamics of metastasis various cancer cells including fibrosarcoma, melanoma, breast, and bladder (126).

The study of PDGFRα and cellular motility is well-suited for the chorioallantoic membrane model in that it is immune deficient and is well vascularized. We could assess cell lines lacking or expressing PDGFRα, probing for the relevance of individual pathways/genes that may be upregulated in the PDGFRα-induced EMT process, as identified in this thesis. For example, particular genes such as *SPP1* and *LPAR4* are significantly upregulated with PDGFRα expression and may be critical components needed for the increased motility and invasion potential. These genes could be knocked down in PTC cells and compared to the controls following their inoculation into the chick embryo, to assess for invasive potential in a microenvironment that more closely resembles physiology than simple cell culture.

6.5 Targeting PDGFRα to reverse resistance to radioactive iodine in papillary thyroid carcinoma and improving patient outcomes

We show for the first time that the loss of TTF1 expression is central to the mechanism of PDGFRα-initiated dedifferentiation in PTC. In transformed benign thyroid follicular cells, as well as immortalized thyroid cancer cell lines of varying derivations, PDGFRα induced more invasive phenotypes lacking iodide transport. PDGFRα disrupted the crucial role of TTF1 required in the function of a mature follicular cell as very well documented by work in embryonic stem cells (88). During early-to-mid embryogenesis, groups of cells that are destined to become the follicular cells are set apart by the co-expression of four transcriptional factors - PAX8, TTF1,

HEX, and TTF2, which coordinate a thyroid-specific gene expression program (88). Well-differentiated follicular cells are characterized by the expression of these thyroid-specific genes including those for thyroglobulin (TG), thyroid peroxidase (TPO), sodium iodide symporter (NIS), and the thyroid-stimulating hormone receptor (TSHR). Sustained expression of these genes and maintenance of the differentiation status is dependent on the coordinated action of the transcriptional regulators (89). In fact, in the embryonic stem cell study, when overexpressed transiently, TTF1 alone sufficiently drove embryonic stem cell differentiation towards the follicular cell lineage, as the gene expression levels of thyroid-specific markers NIS, TSHR, TG, and TPO increased. In contrast, PAX8 alone had marginal effects on the expression of these markers, and only enhanced differentiation when co-expressed with TTF1 (88). This dataset might represent a plausible explanation for the observations we report here - that the dedifferentiation cue from PDGFRα effectively suppressed iodide uptake as a result of its downregulation of TTF1 expression, even though PAX8 expression was unaffected.

Resistance to RAI therapy is ultimately due to the loss of NIS expression/activity, which was a not a well understood process (90). We have shown that PDGFRα is a specific and strong downregulator of TTF1 expression with a consequent decrease in iodide transport through disrupted NIS production. This is an important phenomenon, as 30% of metastatic differentiated thyroid cancer (of which the majority are PTCs) exhibit some degree of dedifferentiation (91). The result is the loss of thyroid-specific functions such as the ability to accumulate iodide.

Consequently, these tumors can no longer be responsive to standard radioiodine (RAI) therapy (92). The ability to accumulate RAI is strongly associated with the prognosis in PTC patients. Besides age, RAI avidity is the most significant factor that accounts for survival rates in patients with metastases (93-95). Clinically, RAI-refractory metastatic PTCs are associated with more aggressive growth and metastatic spread (92). We investigated the role of PDGFRα in PTC cell dedifferentiation and acquisition of RAI therapy-resistance.

It was evident from our patient specimens and data that the clinical outcomes associated with PDGFRα-positive papillary thyroid tumors corresponded with those exhibited by dedifferentiated tumors. Patients harboring tumors with PDGFRα showed higher risk of metastasis and recurrence, reduced TTF1 expression, and required significantly higher doses of RAI, relative to patients lacking PDGFRα tumor expression. The clinical relevance of this discovery is that PDGFRα has the potential to predict resistance to radioactive iodide, and this could be used to inform decisions regarding prophylactic node dissection in PTC.

The first therapeutic strategy, re-differentiation, attempts to induce endogenous NIS expression taking advantage of the fact that the NIS gene is not usually defective in PTC cells (90). This is expected to enhance RAI uptake and permit effective ablation of thyroid remnants and metastatic deposits. Retinoic acid analogues were the first to be widely studied for their potentials as re-differentiation agents. In many of the studies, only a few patients with the refractory disease responded to the treatment with elevated radioiodide uptake (96, 97). Further analysis

also showed that increased RAI uptake with retinoic acid analogues did not always correlate with clinical response (92). Due to the limited benefits, their routine use is yet to be recommended. Another promising re-differentiation agent, thiazolidinedione, was shown to have both re-differentiation and anti-proliferative effects on thyroid cancer cells *in vitro* (98). Other agents including histone deacetylase inhibitor, DNA methylation inhibitors and arsenic trioxide, have shown differentiation inducing properties by increasing NIS expression and radioiodide uptake *in vitro* (99). Further studies to evaluate clinical application, dosage, duration, and combination regimen for these re-differentiation agents are still required (99).

Secondly, with improved understanding of the molecular events that trigger the development and progression of PTC, many new molecular targeted drugs are being examined under investigational use in patients with the RAI-refractory disease (99). Since hyperactivity of the MAPK/Erk and PI3K/Akt pathways is believed to be precipitating events in PTC development and progression, the second strategy takes advantage of this pathology by targeting participants in these pathways. These targeted drugs, including TKIs, also inhibit an array of tyrosine kinases known to activate these pathways aberrantly in PTC (99).

The central challenge to advancing TKI therapies for metastatic, RAI-resistant PTC is how to improve drug selection and efficacy, while minimizing toxicity (100). The FDA approved ones, sorafenib and lenvatinib, inhibit multiple tyrosine kinases driving thyroid carcinogenesis and progression. Patients who received lenvatinib under clinical trials had more adverse effects at 97%, compared to 60% in the placebo

group. Adverse effects of special interest associated with lenvatinib treatment include hypertension, proteinuria, arterial thromboembolic effects, venous thromboembolic effects, renal failure, hepatic failure and gastrointestinal fistula. Also, 6 out of the 20 deaths that occurred within the lenvatinib group were considered drug-related (101). Common adverse outcomes observed with sorafenib treatment include hand–foot skin reaction (76·3%), diarrhoea (68·6%), alopecia (67·1%), and desquamation (50·2%) (102).

The safety concerns and mixed outcomes from the trials can be attributed to the fact that these drugs target PDGFRα in the mM range and thus may only be partially effective with increased side-effect profiles (103, 104) compared to an agent targeting only PDGFRα. Tyrosine kinases affected by sorafenib include BRAF, VEGFRs 1 - 3, platelet-derived growth factor receptor (PDGFR) -β, and RET, c-kit and Flt-3 (60, 106). Lenvatinib has the capacity to inhibit VEGFR 1 - 3, fibroblast growth factor receptors (FGFRs) 1–4, RET, c-KIT, and PDGFRs (105, 106). While it is clear that some of these kinases have a role in thyroid tumorigenesis, the subset of thyroid cancers most likely to generate metastases and poor outcomes are dedifferentiated tumors (92). Thus, without their ability to alter thyroid follicular cell differentiation, therapies developed against these tyrosine kinases will not have durable effects on PTC tumor progression. Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase previously reported to promote dedifferentiation in normal thyroid follicular cells. EGFR down-regulated TG and TPO expression (107, 108), and this resulted in repressed iodide uptake (107, 109). However, there is no

information on EGFR expression levels in cancer cells as well as patient samples, and its potential utility in the clinical management of RAI-refractory metastatic PTC is unknown.

Thus in a series of future experiments, we will examine the effect of blocking individual tyrosine kinase receptors on iodide uptake *in vivo*, using a SCID mouse model combined with [18F]TFB - PET imaging machinery. In this way, we can monitor the function of the iodide symporter alongside tumor growth in real time, with varying inhibitor concentrations and treatment durations. However, initial experiments will be performed in cell lines that lack or express PDGFRα as well as patient tumors isolates from primary tumors or metastatic deposits. This way, we could systematically chart the sensitivity of tumors to certain drugs, not only in terms of changes in growth potential but also in terms of iodide transport.

Here, we provide the first demonstration that PDGFRα promotes dedifferentiation by decreasing TTF1 expression in the nucleus, which in turn suppresses iodide transport in thyroid follicular cells. Our study further presents a proof of principle that selectively blocking PDGFRα signaling could significantly improve radioactive iodide treatment and decrease metastases in PTC. This selective therapy should minimize side effects compared to drugs that block multiple tyrosine kinase receptors and improve patient outcomes. Therapies, which enable direct inhibition of PDGFRα (TKIs or antibody-mediated), are currently in clinical trials for the management of other malignancies with aberrant expression of this receptor (110). Crenolanib, a TKI, which inhibits PDGFRα with high potency, was used in this

study (111). Crenolanib augmented iodide uptake and abrogated the aggressiveness associated with PDGFRα expression/activation in cell line models. Its anti-tumorigenic activity was further confirmed in mouse xenograft models. Crenolanib is currently undergoing preclinical and clinical studies in GISTs, gliomas and non-small-cell lung cancers (111-113). Olaratumab, a neutralizing antibody against PDGFRα (114-116) is currently in phase III clinical trials for the treatment of advanced or metastatic soft tissue sarcoma. MEDI-575 another antibody against PDGFRα, also yielded promising results in a Phase I study of patients with advanced solid tumors (117).

Taken together, the body of evidence presented in this thesis strongly supports a central role for PDGFRα in the nodal spread of papillary thyroid tumors. We have compelling indications of the pro-metastatic consequences of PDGFRα signaling in PTC cells, including the elevated expression of EMT-inducing factors, invadopodia formation, increased ECM degrading activity, increased motility, and downregulation of TTF1 expression, which resulted in impaired radioiodine avidity. A graphic summary of the findings presented in this thesis with proposed translational implications are summarized in **Fig. 6.1**.

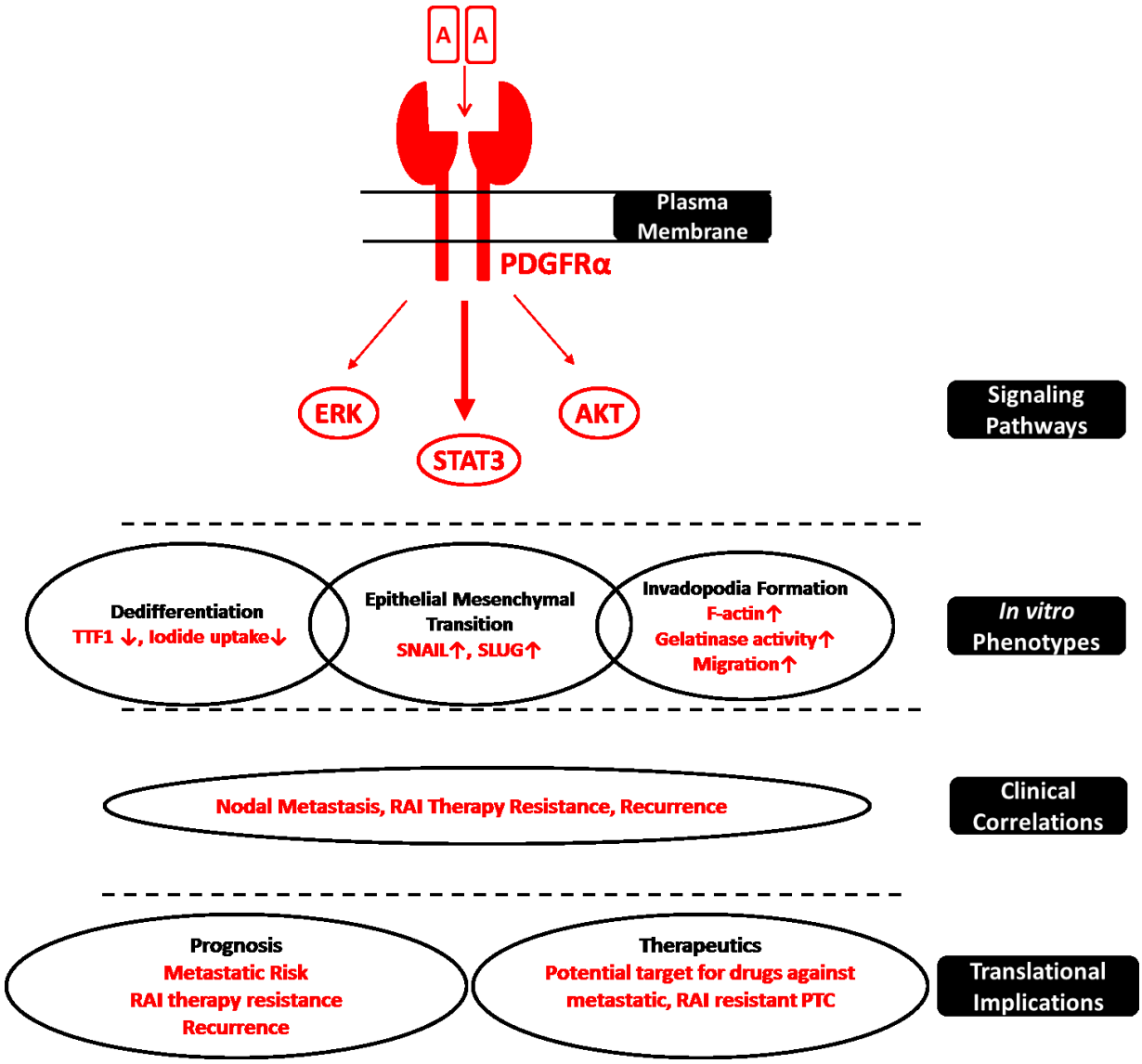

Figure 6.1: A graphic summary of the signaling, phenotypic and clinical consequences of PDGFRα signalling in papillary thyroid cancer, as presented in this thesis.

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