The Role of Platelet Derived Growth Factor Receptor in Neuroendocrine Tumours

by
Yi Man Ko

Master of Science
In
Experimental Surgery

Department of Surgery
University of Alberta

Abstract

Neuroendocrine tumours (NETs) are composed of a broad family of tumours that develop from neuroendocrine cells dispersed throughout the body. The most common of which are carcinoid and pancreatic NETs. While NETs are relatively rare, the estimated incidence of NETs in the United States is increasing since the 1970s. Prognosis following complete surgical resection for local-regional disease is quite favorable. However, most patients present with metastatic disease making curative resection unlikely. Currently, there are limited therapeutic options in the setting of metastatic disease. NETs are highly vascular and angiogenesis plays an important role in tumour progression. Platelet-derived growth factor and its receptor (PDGF/PDGFR) is a class of growth factors which plays a significant role in angiogenesis. However, its role in NETs is not completely understood. The purpose of our study was to: i) assess the expression of PDGFR-α in human pancreatic neuroendocrine cell lines BON-1 and QGP-1 as well as in clinical specimens, ii) to determine the effect of PDGFR-α expression on cell migration and proliferation, and iii) to determine the effect of PDGFR-α expression on tumor growth in mouse xenograft model. We found that, although PDGFR-α is expressed on clinical specimens, neither BON-1 nor QGP-1 cell line express PDGFR-α. Therefore, stable BON-1 and QGP-1 constructs with inducible PDGFR-α expression were established to characterize its role in promoting tumour metastasis in NETs. The activation of PDGFR-α in BON-1 enhanced its migratory potential and the transformed phenotype can be reversed by selective PDGFR-α blockade. For both BON-1 and QGP-1, PDGFR-α expression increased tumor angiogenesis *in vivo*. Based on our results, tyrosine kinase inhibitors that target PDGFR, specifically the α-subunit, may be better suited to treating NETs.

Acknowledgements

First and foremost, I wish to express my sincerest gratitude to my supervisor Dr. Todd McMullen who demonstrated a spirit of adventure in regard to research and scholarship. Without his constant encouragement, support and guidance, this dissertation would not have been possible. I would also like to thank my committee members, Dr. Sandy McEwan and Dr. David Murray for their feedback and support.

I would also like to extend my heartfelt appreciation to my laboratory manager, Ana, for her technical support and teachings during the last two years.

Thank you to the Department of Surgery and Clinical Investigator Program for providing financial support during my graduate study. I would also like to express my gratitude to Dr. David Bigam (Director of CIP) for his ongoing encourage, support, and feedback.

Last but certainly not least, I must acknowledge my family for their ongoing love, patience, and support. They have instilled many admirable qualities in me, and have taught me about staying positive and optimistic despite challenges, and to persevere towards my life goals while never forgetting to maintain a healthy work-life balance. With the completion of this dissertation, I now look forward to starting a new chapter of my life!

Table of Contents

List of Figures

Chapter 1: Introduction

1.1. Introduction to Gastroenteropancreatic Neuroendocrine Tumours (GEP-NETs)

1.1.1. Historical Perspective

A major breakthrough in the understanding of the true extent of the endocrine system was the discovery of hormone-producing cells dispersing throughout a wide variety of non-endocrine tissues and organs. Such cells were first described in the intestine by Heidenhain in 1870 based on their affinity for staining with chromium salts. The similar observations were then independently confirmed by Kulchitsky in 1897. In 1914, Masson further demonstrated that these cells also stain readily with silver salts [1, 2]. In addition to their presence in the gastrointestinal tract, cells with similar histochemical features were also identified in other sites such as the lung, thymus and thyroid gland. All of these observations ultimately provided the foundation for the concept of a disseminated endocrine system that could exhibit either local or systemic effects [3].

Pearse discovered in the late 1960s that many of these cells have the ability to import as well as decarboxylate amine precursors [4]. Hence, these cells were subsequently termed the APUD (*a*mine *p*recursor *u*ptake and *d*ecarboxylation) cells. In addition to its capacity for amine uptake and decarboxylation, APUD cells were found to contain high density of membrane-bound secretory granules which contain mostly peptide hormones of varying physiologic functions [1]. It is the presence of these secretory granules that allows for the accurate identification of these APUD cells in vitro. Based on the similarities of APUD cells to cells of known neural differentiation, it was initially postulated that APUD cells originated from the neural crest. The

term "paraneuron" was introduced to describe these cells. Nonetheless, embryologic studies utilizing the chick-quail chimera system soon refuted the postulated neural crest origin of the majority of APUD cells [5]. In fact, it has been demonstrated that these APUD cells develop from multipotent stem cells within the intestinal crypts and pancreatic ductal epithelium [1]. However, little is known about the factors responsible for the transformation of multipotent cells into APUD cells. So far, 15 cell types with APUD characteristics have been identified and are often referred to as the neuroendocrine cells [1, 6]. The term "neuroendocrine" is a misnomer in that it does not imply any embryologic connection of these cells to neuroectoderm, but rather is meant to reflect the shared phenotype characterized by the presence of genes encoding both endocrine and neuronal features [1].

1.1.2. Histopathological Classifications of GEP-NETs

Traditionally, Williams & Sandler (1963) classified GEP-NETs according to the embryonic site of origin into foregut (gastric, duodenal, and pancreatic), midgut (jejunal, ileal, appendiceal, and cecal) and hindgut (colonic and rectal) tumours [2, 3, 7]. However, within these subgroups the clinical and biological features vary considerably and the usefulness of this classification in practical diagnostic work is limited [8, 9]. A major milestone in the diagnostic approach and management of GEP-NETs has been their classification by the anatomic site of origin. In addition, within the last decade, it has become apparent that GEP-NETs have different inherent characteristics according to the degree of differentiation or grading [10].

Although many different classification criteria have previously been used, the one introduced by European Neuroendocrine Tumour Society (ENETS) has recently been implemented from the World Health Organization (WHO) [11]. According to this classification system, high grade (G3) tumours (mitotic count of more than 20 per 10 high-powered fields or a Ki-67 proliferation index of more than 20%) are distinctive from the low-to intermediate-grade (G1 and G2) tumours. Both G1 and G2 GEP-NETs are considered well differentiated tumours whereas G3 characterizes poorly differentiated tumours. The GEP-NETs that show non-endocrine components (exceeding at least 30% of all tumour cells) are called mixed adenoneuroendocrine carcinomas (MANEC) [11].

1.2. Molecular Biology of GEP-NETs

1.2.1. Genetic syndromes associated with GEP-NETs

The estimated incidence of GEP-NETs in the United States was 5.25 cases per 100,000 in 2004 according to the SEER database, suggestive of an increasing incidence [6]. While most GEP-NETs occur sporadically, they may arise in the setting of inherited genetic syndrome including multiple endocrine neoplasia (MEN), von-Hippel-Lindau (VHL) disease, tuberous sclerosis complex (TSC), and neurofibromatosis (NF) [12, 13].

1.2.2. Multiple Endocrine Neoplasia (MEN)

The MEN syndromes are characterized by tumours that mainly affect the endocrine organs. There are two types of MEN, namely the MEN 1 and MEN 2. MEN 1 is an inherited autosomal dominant syndrome affecting primarily the parathyroid glands, pituitary gland, and the endocrine pancreas. In addition, MEN 1 is also associated with multiple GEP-NETs. MEN 2, on the other hand, also is an inherited autosomal dominant syndrome which is associated with medullary thyroid cancer (MTC), pheochromocytoma (often bilateral disease), and hyperparathyroidism. The underlying genetic perturbation in MEN 1is the inactivation of tumour suppressor gene *MEN 1* (chromosome 11q13 encoding the menin protein), whereas MEN 2 is associated with germline mutation of the RET proto-oncogene (chromosome 10q11.2) which leads to activation of the tyrosine kinase receptor RET [12, 13].

1.2.3. Neurofibromatosis (NF)

NF (also known as von Recklinghausen disease) shows an autosomal-dominant inheritance with high penetrance. The underlying genetic mutation in NF1 is the inactivation of tumour suppressor gene *NF1* (chromosome 17q11.2 encoding protein neurofibromin). In most patients, there is sufficient clinical evidence of the disorder to allow early diagnosis. The genetic syndrome is characterized by neurofibromas, *Café au lait* patches of the skin, and, bone dysplasia. GEP-NETs are rare and occur in 1% of the NF patients. Most of the GEP-NETs are concentrated in the peri-ampullary region of the duodenum [12, 14].

1.2.4. von Hippel-Lindau Syndrome (VHL)

VHL disease a dominantly inherited familial cancer syndrome caused by germline mutations in the VHL tumour suppressor gene (chromosome 3p25). The prevalence of GEP-NETs in VHL patients has been reported at frequencies of 5–17% and are usually confined to the pancreas. Clinically, in approximately 30-50% of VHL patients, the tumours are multifocal and almost all of the tumours are functionally inactive [12, 15].

1.2.5. Tubular Sclerosis Complex (TSC)

TSC is an autosomal-dominant genetic disorder with a disease-penetrance of approximately 100%. TSC is a multi-organ disorder displaying a wide range of clinical manifestations characterized by formation of hamartomatous lesions. TSC is caused by inactivating mutations in either the TSC1 gene (chromosome 9q34) or the TSC2 gene (chromosome 16p13.3) encoding for proteins hamartin and tuberin, respectively. GEP-NETs have rarely been described in TSC patients with a prevalence of < 1% and the lesions are often confined to the pancreas [12, 16, 17].

1.2.6. Molecular genetics of GEP-NETs

The genetics of tumourigenesis in GEP-NETs have yet to be fully deciphered. However, several studies utilizing comparative genomic hybridization (CGH) have demonstrated frequent allelic deletion of chromosome 18 in intestinal NETs [12, 13, 18, 19]. On the other hand, the most

frequent abnormality in pancreatic NETs is the deletion of chromosome 11q, the location of the MEN1 gene [20]. Nonetheless, the majority of GEP-NETs is sporadic.

The p53, a tumour suppressor protein, is encoded on chromosome 17. Normally, the wild type p53 has such a short half-life that it is virtually impossible to be detected by immunohistochemical techniques. However, most mutant p53 have longer half-lives and are readily detectable. With regard to GEP-NETs, p53 mutations have only been described in atypical pulmonary carcinoid [18, 19, 21].

The Bax and Bcl-2 proteins play an important role in mediating apoptosis. The Bax protein indeed shares 21% homology with the Bcl-2 protein. Several studies have investigated the expression of Bax and Bcl-2 in pulmonary NETs. Many have reported over-expression of Bcl-2 and down-regulation of Bax in the more aggressive form of NETs [21, 22]. In gastric NETs, Bcl-2 has been implicated as an early oncogene [19].

1.3. Diagnosis of GEP-NETs

1.3.1 Incidence and Prevalence of GEP-NETs

GEP-NET disease is increasing in incidence partly because of increased usage of cross-sectional imaging modalities as well as physician awareness. According to the most recent SEER registry (SEER 17), greater than half of all neuroendocrine tumours (61.0%) are GEP-NETs, with the highest frequency in the rectum (17.7%) followed by the small intestine (17.3%) and colon

(10.1%). Appendiceal, gastric and pancreatic NETs account for 7.0%, 6.0%, and 3.1% of NETs, respectively. The 5-year survival rates differ depending on the location of the primary tumour, and each primary tumour site has a distinct epidemiologic profile. For example, rectal NETs are often diagnosed at a younger age with good prognosis, whereas colonic NETs are usually diagnosed at an older age with poor survival. The overall 5-year survival rate of GEP-NETs is 68.1%. Among all, pancreatic NETs have the lowest 5-yr survival rates (37.6%) and rectal NETs have the highest 5-year survival rates (88.5%) [23, 24].

1.3.2. Clinical recognition of GEP-NETs with and without carcinoid syndrome

Patients with GEP-NETs may or may not have symptoms attributable to hormonal secretion. These hormones can cause debilitating symptoms such as flushing, diarrhea, and congestive heart failure in patients with carcinoid syndrome and symptoms attributable to secretion of insulin, glucagon, gastrin, and other peptides in patients with pancreatic NETs. Patients with hormonal symptoms are considered to have “functional” tumours, while those without symptoms are considered to have “non-functional” tumours [7, 10, 25].

GEP-NETs have the capacity to secrete bioactive substances most notably, 5-Hydroxytryptamine (5-HT), histamine, and various kinin peptides [26, 27]. These substances are responsible for the constellation of symptoms, which includes diarrhea, flushing, and organ fibrosis, known as the carcinoid syndrome once released into systemic circulation without first being metabolized by the liver [28, 29]. Under normal circumstances, the release of these bioactive substances causes little if any symptoms because of first-pass metabolism by the liver [28]. However, with large

tumour burden or presence of hepatic metastases, the liver's ability to inactivate these substances may be overwhelmed and may consequently result in carcinoid syndrome. Primary NE tumours that drain directly into the systemic circulation such as bronchial, ovarian, or testicular tumours are also capable of producing carcinoid syndrome because they bypass metabolism in the liver [29].

About 10% of patients with a GEP-NET develop the classic carcinoid syndrome. Carcinoid syndrome has a clinical spectrum that includes hypotension or hypertension, cutaneous flushing, bronchoconstriction, diarrhea, intestinal obstruction and carcinoid heart disease [26]. These symptoms may have a paroxysmal onset and vary in severity.

The host of functional entities in GEP-NETs also includes functional pancreatic NETs which are capable of secreting numerous hormones each manifesting in a distinct clinical presentation. Insulinomas are the most common pancreatic NET, comprising 30–45% of these tumours. However, they remain a rare clinical entity with an estimated incidence of 0.4/100,000 population [30, 31]. Similar to their normal beta-cell counterparts, insulinoma cells release pro-insulin composed of C-peptide and insulin causing symptomatic hypoglycemia.

Gastrinomas are the second most common functional pancreatic endocrine tumour with an incidence of 1-2 per million population. Gastrinomas cause the Zollinger-Ellison syndrome (ZES), a condition in which there is increased production of the hormone gastrin (normally synthesized by G cells located in the antral mucosa of the stomach) [30, 32, 33]. Gastrin is a peptide hormone that stimulates secretion of gastric acid by the parietal cells of the stomach and

aids in gastric motility. The primary clinical manifestations of gastrinoma are recurrent peptic ulcer disease and unexplained secretory diarrhea.

VIPomas are rare pancreatic NETs which release high levels of vasoactive intestinal polypeptide (VIP) producing the clinical syndrome of watery diarrhea, hypokalemia, and achlorhydria also known as the Verner–Morrison syndrome or pancreatic cholera [33-36]. Overall, these tumours are exceedingly rare with an incidence of less than 1 per 2 million population. Glucagonoma is another rare islet cell tumour with an incidence of less than 1 per million population. It is believed to arise from neoplastic alpha-cells, which normally produce glucagon to maintain glucose homeostasis. Glucagonoma releases large amounts of glucagon, which induces a state of catabolism and its presentation is one that may include dermatitis (necrolytic migratory erythema, NME), diabetes, depression (or confusion), and deep vein thrombosis [36, 37].

Lastly, somatostatinomas are exceedingly rare with an estimated incidence of 1 per 40 million population [33, 36, 38]. Most somatostatinomas (68%) are found in the pancreas (usually the head) with the remainder in the duodenum or elsewhere in the small intestine. Pancreatic somatostatinomas present with symptoms of excessive somatostatin activity also known as the "somatostatin syndrome" [38]. The clinical manifestations are caused by the inhibitory effect of somatostatin on exocrine and endocrine secretory organs of the gastrointestinal tract.

1.4 Biochemical assessment of GEP-NETs

1.4.1. Overview

GEP-NETs upon specific stimulation secrete hormones mediating various functions [39]. The diverse neuroendocrine cell system can secrete products, such as peptides and biogenic amines that are tumour specific and may aid in the disease diagnosis as well as treatment follow-up. Some tumour markers may even have prognostic implications [40-43].

The chromogranins constitute a large family of glycoproteins, of which Chromogranin A (CgA) and Chromogranin B (CgB) have attained most clinical interest [41, 44]. Chromogranins are found in neuroendocrine cells throughout the body, but are also located in the neuronal cells in the central and peripheral nervous systems [41, 42]. GEP-NETs often present with increased plasma levels of CgA and sometimes also CgB [41, 42, 44, 45].

1.4.2. Chromogranins

CgA is the most widely used general biomarker for the diagnosis of GEP-NETs as it is co-secreted by the majority of neuroendocrine cells and the plasma level of CgA persists even after malignant transformation [46-48]. The most reliable and robust immunoassays for tumour detection have been those that measure the whole CgA molecule [42]. In comparison, assays targeting specific parts of the molecule usually have lower sensitivity [49]. Commercial kits for determining CgA are available and measurement of CgA is routine in the management of GEP-

NETs [42]. However, it should be noted that there is a fair amount of variability among different assays with sensitivities ranging from 67 to 93% [42, 44]. In most GEP-NETs, CgA is more abundant than CgB and therefore it is usually a better circulating tumour marker than is CgB.

1.4.3. Pancreatic Polypeptide

Pancreatic polypeptide (PP) is a 36 amino acid linear-peptide and is secreted primarily by PP cells located in the gut mucosa and pancreas. Pathologically, elevated levels of PP have been detected in GEP-NETs, with an overall sensitivity of about 50–80% [42, 43]. However, there are many conditions such as diarrhea, laxative abuse, and chronic kidney disease where levels of PP can be falsely elevated [42]. When CgA and PP are combined, the sensitivity for diagnosis of GEP-NETs increases to 95% [42].

1.4.4. Neuron-Specific Enolase

Neuron-specific enolase (NSE) is a cytoplasmic protein present in neurons and neuroendocrine cells. It serves as a circulating marker for NETs. In patients with poorly differentiated NETs, NSE might be elevated despite normal CgA measurement. The magnitude of NSE elevation also correlates roughly with tumour size, although the specificity is lower than that of CgA [50, 51].

1.5. Anatomic Imaging of GEP-NETs

1.5.1. Cross-Sectional Imaging

Cross-sectional imaging plays an important role in the disease localization, the detection of metastases, and evaluating treatment response. Computed tomography (CT) is currently the main modality, although magnetic resonance imaging (MRI) is catching up in recent years with regard to detection rate. Pancreatic NETs are often small at the time of diagnosis and appear isodense with the pancreatic parenchyma on pre-contrast images. However, pancreatic NETs are usually hypervascular, and detection of both the primary lesion as well as liver metastases can be optimized using intra-vascular contrast medium [52]. Most pancreatic NETs enhance avidly following administration of contrast medium and are visualized during the early arterial phase of imaging with washout during later venous imaging. The overall sensitivity for CT detection of the primary pancreatic NETs is about 60-80%. In contrast, the detection rate of CT for primary intestinal NETs is less ideal. Despite technological advances, detection of small primary lesions in small bowel remains one of the most difficult challenges [53]. More commonly, CT imaging for intestinal NETs often demonstrates liver metastases and secondary features such as kinking and tethering of the bowel mesentery caused by tumour-associated desmoplastic fibrosis.

1.5.2. Endoscopic Ultrasound

Endoscopic ultrasound (EUS) is often used to evaluate both solid and cystic lesions in pancreas and is particularly useful in the diagnosis of pancreatic NETs when utilized in conjunction with

fine-needle aspiration (FNA). In addition, EUS also provide additional information such as relationship of the lesion to the pancreatic duct for operative planning. EUS is capable of identifying small (<1cm) pancreatic NETs with an overall sensitivity of 90% [53].

1.5.3. Nuclear Imaging (Receptor-Based Functional Imaging and Metabolic Imaging)

The methods for nuclear imaging of GEP-NETs can be divided into two main categories, namely tracers that are based on selective expression of receptors and metabolic signature of the lesion. The presence of somatostatin receptors (SSTRs) on many GEP-NETs is the foundation for the development of somatostatin receptor scintigraphy (SRS). The majority (>80%) of GEP-NETs expresses high densities of SSTR (especially subtype 2 and 5). Radio-labeled synthetic analogs of somatostatin can therefore be exploited to identify and locate these lesions. Alternatively, activation of specific metabolic pathways used in the peptide synthesis unique to GEP-NETs provides another possibility for targeted imaging [54].

SRS is currently the method of choice for the staging of GEP-NETs [55]. Somatostatin is a small regulatory peptide, which is widely distributed in the human body. It has an inhibitory effect on the production of various exocrine hormones in the gastrointestinal tract [56]. SSTRs are G-protein-coupled receptors capable of activating signal transduction pathways upon binding with an extra-cellular ligand. Thus far, six different somatostatin receptors have been identified, namely SSTR1-5 (SSTR2 can be alternatively spliced to yield SSTR2A and SSTR2B).

GEP-NETs often express large amount of SSTRs on their cell surface, which can be exploited by imaging techniques using radio-labeled somatostain analogues. The endogenous somatostatin has a very short plasma half-life, about 3 minutes. Therefore, synthetic somatostatin analogues with longer half-life such as octreotide, vapreotide, and MK678 have been developed. Currently, radio-labeled analogues of these synthetic somatostatin are all in clinical use for imaging [55, 57]. These synthetic somatostatin analogues all have strong affinity for SSTR2 and SSTR5, but with varying binding to SSTR3 and SSTR4. Depending on the chelators, namely DTPA or DOTA, that are coupled to the somatostatin analogues, different radio isotopes can be attached allowing for different imaging techniques. For example, when somatostatin analogues are labeled with 111In or 99mTc, they can be used for scintigraphic purposes. On the other hand, when labeled with positron emitting isotopes, such as 18F, 64Cu or 68Ga, the somatostatin analogues can then be used for PET imaging [57-60].

SRS is now readily available, and the overall sensitivity in the detection of GEP-NETs is about 88%. The advantage of PET imaging over conventional scintigraphy is that PET imaging offers a better spatial resolution and the imaging quality is not dependent on tracer-receptor complex internalization [56, 60, 61]. Results from the studies indicate a sensitivity and specificity of 97% and 92%, respectively, for the detection of GEP-NETs [59-61].

The ability of GEP-NETs to synthesize and secrete many different peptides distinguishes themselves from other malignancies. The abnormally activated metabolic pathways necessary for the production of these tumour-specific peptides become ideal candidates for the development of specific tracers. These pathways can be targeted at multiple levels and one of the most studied

pathways is the catecholamine pathway. In general, tracers can be developed as a marker for the transporter proteins, as a substrate for the pathway, or as a marker for the uptake of end-product [62].

In the catecholamine pathway, phenylalanine and L-DOPA are taken up via the large amino acid transporter (LAT) system into the cell [62]. Within the cytoplasm, these precursors are metabolized to dopamine, which is transported and stored into secretory vesicles via the vesicular monoamine transporter (VMAT) system [63]. Once secreted into the extra-cellular space, these end products can be recycled via the norepinephrine re-uptake transporter. Tracers developed for this pathway include the precursor, 6-18F-l-3,4-di-hydroxyphenylalanine (18F-DOPA), for the LAT system as well as the substrate, metaiodobenzylguanidine (MIBG), for the norepinephrine re-uptake transporter [64, 65].

18F-DOPA is a variant of L-DOPA labeled with radio-isotope 18F designed specifically for PET imaging. It acts as a precursor for the catecholamine pathway. Despite having an 18F atom in L-DOPA, it has minimal influence for the transport of 18F-DOPA into the cytoplasm via the cell membrane LAT system [66]. Within the cytoplasm, 18F-DOPA is preferentially decarboxylated to 18F-dopamine at a faster rate than L-DOPA by the enzyme aromatic acid decarboxylase (AADC). The resultant 18F-dopamine is then transported into secretory vesicles by VMAT system. Thus far, there are only a few studies on 18F-DOPA as a tracer for the detection of GEP-NETs. Expectedly, 18F-DOPA PET imaging yields a very high sensitivity, particularly in the detection of paragangliomas and pheochromocytomas. The overall sensitivity of 18F-DOPA PET for detection of GEP-NETs is averaging 89% [65, 67-69].

MIBG is an iodinated guanethidine variant, structurally resembling the endogenous amine norepinephrine. In contrast to 18F-DOPA, MIBG acts as a substrate for the norepinephrine re-uptake transporter. The precise uptake and retention mechanism of MIBG in GEP-NETs is not fully understood. However, it appears that the norepinephrine re-uptake transporter plays an important role [62]. MIBG can be labeled with either 123I or 131I. However, 123I labeled MIBG is the tracer of choice for diagnostic imaging applications. It has several advantages over 131I-MIBG, namely lower radiation burden and superior image quality [70].

MIBG scan has been shown to be highly sensitive and specific for detecting NET arising from adrenal medulla, but may also be taken up by other GEP-NETs. MIBG scan has an overall sensitivity of 90% in detecting pheochromocytomas, paragangliomas and neuroblastomas. However, its diagnostic accuracy for the detection of other GEP-NETs is much lower averaging 50%.

Despite advances in imaging modalities, clinicians still routinely encounter patients with lesions that are not detected by either the somatostatin or the MIBG scan. It is imperative that new diagnostic markers are to be explored.

1.6. Surgical management of GEP-NETs

1.6.1. Overview

Complete surgical resection is the only curative therapy for patients with GEP-NETs. However, GEP-NETs often metastasize to the liver before they are diagnosed making curative resection unlikely [71]. Standard surgical therapy involves resection of the primary lesion with draining nodal basin and/ or distant metastases with curative intent [72]. Cytoreductive procedures for symptomatic relieve may also be undertaken but overall the surgical options are varied depending on the site and stage, as briefly outlined below.

1.6.2. Gastric NETs

There are 3 types of gastric NETs, namely type I-III. Type I gastric NETs is the most common form (~70%) which is associated with chronic atrophic gastritis and pernicious anemia. Type II gastric NETs (5-10%) is associated with MEN1 or Zollinger-Ellison syndrome. Type III (~15%) gastric NETs is a sporadic disease that has the highest rate of metastasis and thus the worst prognosis. Both type I and type II diseases are associated with hypergastrinemia whereas the gastrin level in type III is usually not elevated [32, 70].

Type I and II gastric NETs are often small and multi-focal. Endoscopic resection with regular surveillance is an acceptable form of treatment. Lesions larger than 3cm are more aggressive and are associated with higher rate of metastasis. These lesions should be surgically resected

following sound oncologic principles [73]. Type III disease is the most aggressive form and it behaves like a gastric adenocarcinoma. These tumours have a higher propensity for early metastasis and tend to be > 2cm at the time of diagnosis. These tumours should be treated with total or sub-total gastrectomy with lymph node dissection [71, 73].

1.6.3. Small bowel NETs

Many of the small bowel NETs are indentified incidentally during an operation for some other reasons such as bleeding and bowel obstruction. Once identified intra-operatively, an effort should be made to thoroughly search for any additional lesions as small bowel NETs tend to be small and multi-focal. The extent of surgical resection should follow standard oncological principles with en-bloc resection of the primary lesion with lymph node dissection [72, 74].

1.6.4. Appendiceal, colonic and rectal NETs

For most appendiceal NETs, simple appendectomy is considered to be curative. However, if the lesion is located at the base, angioinvasion is present, mitotic index is high, or the lesion is greater than 2cm, the rate of metastases are significantly higher and a formal right hemi-colectomy together with lymph node dissection should be performed [75]. In contrast, colonic NETs behave much like adenocarcinomas and proper surgical resection following oncological principles with en-bloc removal of lymph node basin is indicated. For small rectal NETs with no pathologic lymph nodes or evidence of invasion into the muscularis propria on EUS, endoscopic or transanal resection with periodic surveillance is an acceptable form of treatment. In patients

with nodal involvement or invasion into muscularis propria, either a low anterior resection or total mesorectal excision is mandated [72, 76].

1.6.5. Functioning pancreatic NETs

The only cure for insulinoma is complete surgical excision. However, it is often difficult, as with most functioning pancreatic NETs, to localize the lesion pre-operatively. Lately, it has been reported that the detection rates approach 95% when combining intra-operative ultrasound with selective portal venous sampling and arterial calcium injection (83, 84). With better detection rate, pancreas-sparing enucleation has replaced radical pancreatic resection as the surgery of choice [77]. Unlike insulinomas, many of other rare functioning pancreatic NETs such as gastrinomas, glucagonomas and VIPomas are often malignant. Nodal involvement and liver metastasis are common at the time of diagnosis. Radical surgery with curative intent is recommended whenever possible [72, 78]. There is evidence to suggest a role of cytoreductive surgery in symptomatic control.

1.6.6. Non-functioning pancreatic NETs

For patients with non-functioning pancreatic NETs, there is evidence to support an aggressive surgical approach for lesions greater than 2cm [79, 80]. However, the evidence is less clear for lesions less than 2cm, which may be benign. To date, no data exist to support a survival benefit in patients with small non-functioning pancreatic NETs undergoing surgery [81]. The extent of surgery performed depends on the location and size of the lesions. Small lesions (<2cm), mostly

benign, may be treated with local resection such as enucleation or central pancreatectomy. Large lesions (>2cm) or lesions suspected to be malignant should be treated with radical surgery such as pancreaticoduodenectomy or distal pancreatectomy depending on the location of the tumour. Cytoreductive surgery is not indicated for locally unresectable non-functioning pancreatic NETs [72, 79].

1.7. Medical therapy of GEP-NETs

1.7.1. Current outcomes with somatostatin analogues

The basis of using somatostatin analogues for treatment of GEP-NETs lies in the high expression of SSTRs in the majority (>80%) of these tumours [82]. To date, five subtypes of SSTRs have been identified. However, only SSTR2 and 5 are of clinical interest for their ability to inhibit amine and peptide hypersecretion [83]. In fact, the current generation of approved somatostatin analogues binds predominately to SSTR2 and 5.

Traditionally, somatostatin analog therapy has been considered to be useful only for symptomatic control and reduction of biomarker with no proven survival benefit. However, a recent prospective multicenter trial (PROMID) has shown a survival benefit for the use of octreotide LAR (96). In this randomized phase III study, the effect of octreotide LAR on tumour growth in patients with well-differentiated metastatic GEP-NETs (i.e. Ki 67 < 2%) was reported. The study was terminated earlier as the interim analysis showed the median time to disease progression in the octreotide-treated group versus the placebo was 14.3 and 6 months (hazard

ratio of 0.34; $p<0.001$), respectively. Overall, stable disease was achieved in 66.7% of treated and 37.2% of untreated patients [84, 85]. However, subgroup analysis revealed that the benefit of octreotide LAR was most evident in patients whose primary lesion was resected and with less than 10% liver disease. Along with previous clinical data derived mostly from retrospective and non-randomized studies, it is evident that disease stabilization occurs in 35-70% of treated patients [72, 85].

1.7.2. Current outcomes with radionuclide therapy

Peptide receptor radionuclide therapy (PRRT) with radiolabelled somatostatin analogues has been identified as a promising therapeutic option for patients with advanced GEP-NETs [86]. However, the presence of somatostatin receptors on the tumour surface is a pre-requisite for PRRT limiting its clinical application. The basic principle of PRRT in GEP-NETs relies on the high energy β-emitting radio-isotopes to deliver targeted radiation-induced DNA damage to cancerous cells [87]. Several earlier studies have utilized PRRT with the application of different radio-peptides, but the results were quite variable presumably due to heterogeneity in study design, patient population, and delivery methods. To date, the most reputable results were obtained from a single-center experience of 310 patients treated with ^{177}Lu DOTATATE [88]. In this study, patients were treated up to a cumulative dose of 27.8-29.6 GBq, usually in four treatment cycles, with treatment interval of 6 to 10 weeks. Complete response and partial response occurred in 2% and 28% of 310 patients, respectively, and a significant amount of patients also achieved stable disease. Median time to disease progression was 40 months. Median survival from the beginning of treatment and disease diagnosis was 46 and 128 months,

respectively, representing a survival benefit of 40 to 72 months when compared with historical controls. PRRT has a few adverse effects mainly the development of myelodysplastic syndrome and renal toxicity [88]. Despite the impressive clinical outcomes, these results were limited by the non-randomized study design. In another recent study, 90 patients with metastatic intestinal NETs were treated with three cycles of 4.4 GBq of ^{90}Y-edotreotide (once every 6 weeks). Stable disease was achieved objectively in 74% of patient and progression-free survival (PFS) was significantly greater in responding patients (18.2 vs. 7.9 months) [89].

^{131}I-MIBG is another radionuclide agent that has been used for treatment of NETs since the 1970s. Unlike PRRT, ^{131}I-MIBG tends to have better response rate for certain NETs, namely pheochromocytomas and paragangliomas. In the setting of malignant NETs, ^{131}I-MIBG maybe a treatment option for patients with no uptake on octreotide scintigraphy, but with positive uptake on ^{123}I-MIBG scintigraphy. A number of studies from the early 1990s have investigated the utility of ^{131}I-MIBG in NETs, but the results were quite variable. The overall response rate for patients with advanced NETs (stage III/IV) was 26% (ranged from 13% to 39%) [86].

1.7.3. Current outcomes with chemotherapy

Systemic chemotherapy is indicated in patients who are not surgical candidates, who fail to respond to therapy with somatostatin analogues, or who have atypical GEP-NETs with high proliferation rates (proliferation index > 10-20% as evidenced by Ki-67 staining). In general, patients with pancreatic NETs tend to have a better response to systemic chemotherapy than those with intestinal NETs [90]. Historically, responses to single-agent chemotherapy have been

disappointing. Many chemotherapeutic agents such as streptozocin, chlorozotocin, doxorubicin, 5-fluorouracil, taxol, and dacarbazine have been trialed and all are relatively ineffective. Streptozocin as a monotherapy has demonstrated the most activity in pancreatic NETs with response rates ranging from 36 to 42% [91, 92]. Combination therapy, on the other hand, achieved a much higher response rate than monotherapy. The Mayo Clinic has performed one of the first clinical studies on combination therapy comparing streptozocin alone to streptozocin-5-fluorouracil (5-FU) combination in patients with advanced pancreatic NETs and it has achieved an impressive response rate of 63% in the combination regimen [92]. A follow-up study by the same group of investigators also reported a response rate of 69% using streptozocin-doxorubicin combination [93]. However, it is important to note that these response rates from earlier studies may have been positively skewed as a result of reliance on non-radiologic response criteria [92]. More recently, a retrospective review from the MD Anderson reported an objective response rate of 39% using the combination of streptozocin, doxorubicin, and 5-FU [94]. A recent phase II study using the combination of temozolomide and thalidomide also produced an encouraging response rate of 45% in patients with pancreatic NETs as opposed to 7% response rate in patients with intestinal NETs [95]. More recently, the combination therapy using temozolomide and capecitabine has been used in patients with pancreatic NETs as first-line treatment with an impressive response rate of 70%. In this study, patients with metastatic pancreatic NETs were treated with capecitabine (750 mg/m^2 twice daily, days 1–14) and temozolomide (200 mg/m^2 once daily, days 10–14) every 28 days. Among 30 treated patients, 21 (70%) patients achieved an objective radiological response. Median PFS was 18 months and the 2-year survival rate was 92%.

1.7.4. Summary of Treatments

Overall the treatment of NETs is a complicated landscape that may combine surgery with chemotherapy, PRRT, and targeted therapies. Unfortunately, despite this rich collection of different treatment modalities, many patients still do not benefit from the application of one or many of these therapies and continue to develop progressive disease. Especially under circumstances where surgery is unable to control the primary or metastatic disease, there are few options for these patients other than to try to extend their lifespan but there is no hope for cure using the available treatments. Even in patients that have good responses to somatostatin analogues or PRRT, the response may only be transient and again these treatments only slow progression but cannot provide options for cure. Thus it is imperative that we develop novel therapies that can complement our current treatment armamentarium.

1.8. Targeted Therapies

1.8.1. Overview

Cancer cells often have deregulated signaling pathways conferring them with growth advantages. Recently, many molecular targeted therapies have shown promising anti-tumour activity in GEP-NETs as well as many other neoplasias. By selectively interfering with the molecular events involved in tumour development, targeted therapies allow for a more effective, yet less toxic strategy to combat cancer cells than the traditional cytotoxic drugs. These molecularly targeted therapies are known for their specific and highly selective mechanism of actions [96].

1.8.2. Current outcomes with targeted therapy

Angiogenesis has been shown to play an important role in the development of GEP-NETs [83]. Well-differentiated NETs appear to have higher expression of VEGF, PDGF, and microvessel density as compared to the poorly differentiated counterparts [97]. It is this vascular nature of well-differentiated NETs which has led to development of angiogenesis inhibitors as a treatment modality. Sunitinib is an orally available and multi-targeted tyrosine kinase inhibitor that blocks the VEGF receptor, PGDF receptor, KIT and RET. Sunitinib has recently been shown to prolong progression-free survival (11.4 versus 5.5 months) in a phase III study where patients with disease progression were randomly assigned to receive either 37.5mg daily dose of sunitinib or a placebo [98]. In addition, eight objective responses (9.3%) were observed with sunitinib treatment whereas none was described in the placebo arm. Similarly, the mammalian target of rapamycin (mTOR) is a serine-threonine kinase which functions as a common downstream mediator of various signaling pathways implicated in the development of GEP-NETs and it has profound effects on angiogenesis, cellular proliferation and autophagy. Everolimus, the mTOR inhibitor, has been shown to improve progression-free survival (11 versus 4.6 months) in a phase III study where patients with progressive pancreatic NETs were randomly assigned to receive either 10mg daily dose of everolimus or placebo.

Despite the initial success with these molecularly targeted therapies, the clinical reality is much more complex demanding the constant need to identify specific biomarkers that would characterize subgroups of patients who would benefit from these treatments the most. However, to date, no neuroendocrine markers have become available in clinical practice to guide the

preferential therapy. More recently, the expression of several growth factor receptors as well as their downstream signaling pathways has been implicated in the development of GEP-NETs allowing for identification of potential biomarkers for the development of targeted therapy.

1.8.3. Platelet derived growth factors receptors and GEP-NETs

Aberrant expression of platelet derived growth factor (PDGF), and activation of its receptors (PDGFRs) has been implicated in the development of aggressive carcinomas with lymphatic and distant metastases. GEP-NETs also express PDGF and PDGFRs. In fact, expression of PDGF has been found in 70% and 60% of carcinoid and pancreatic NETs, respectively [99, 100]. Clearly, GEP-NETs are capable of secreting a wide array of active growth factors which interact with tumour and its surrounding stroma in a complex autocrine and paracrine fashion. By identifying these key members of the growth factor pathway, novel therapeutic targets may be discovered.

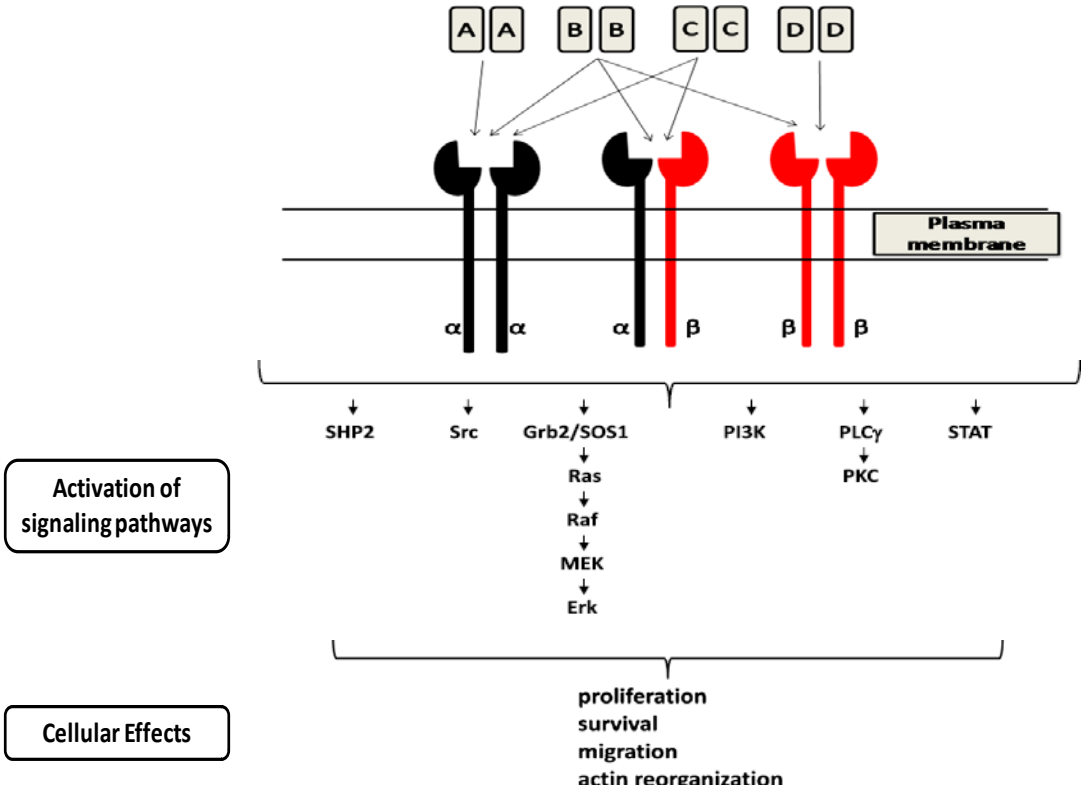


Figure 1.1. Interactions between PDGF ligands and their receptors. The binding of the ligand to its specific receptor initiates receptor dimerization and subsequent autophosphrylation. The resultant conformational changes activate the kinases and expose the docking sites for adaptor proteins for various signalling pathways. Adapted from [101].

PDGFs are a family of peptides that signal through tyrosine kinase receptors (PDGFR with α and β subunits) to stimulate various cellular functions including growth, proliferation, and differentiation. Four different polypeptide chains of PDGF (A, B, C, and D) ligand have been identified. They are linked to form homodimers or heterodimers (AA, AB, BB, CC, and DD). PDGF isoforms exert their cellular effects by binding to PDGFRs. Of interest, PDGF-BB is able to activate all three receptors, whereas PDGF-AA and -DD is specific for PDGFR- α and β, respectively. Upon ligand binding, receptor dimerization occurs and autophosphrylation follows subsequently (Fig. 1.1.).

PDGFR may play a crucial role in regulating cellular processes such as growth and angiogenesis in GEP-NETs. PDGF and its receptors are found to be widely expressed in GEP-NETs, suggesting the need to further evaluate the effect of PDGF-specific monoclonal antibodies as well as tyrosine kinase inhibitors on GEP-NETs [19, 30, 102]. PDGFR may represent a new diagnostic target since a significant percentage of GEP-NETs express this protein. In addition, it may also be a marker for metastases given the known role for PDGFR in angiogenesis as well as the development of lymphatic system. Moreover, PDGFR also represents a potential therapeutic target. The current application of PRRT relies on the expression of somatostatin receptors which in many cases are not found at levels high enough for diagnostic or treatment purposes. Thus, there are many opportunities to identify a role for PDGFR in neuroendocrine tumour biology.

Chapter 2: The Role of PDGFR in Neuroendocrine Tumours

2.1. Introduction

Neuroendocrine tumours (NETs) are composed of a broad family of tumours that develop from neuroendocrine cells dispersed throughout the body [1]. The most common of which are carcinoid and pancreatic neuroendocrine tumours [10]. Other NETs include those arising from the adrenal, parathyroid, pituitary gland, and calcitonin-producing C cells of the thyroid. While most NE tumours occur sporadically, they may arise in the setting of inherited genetic syndromes including multiple endocrine neoplasia (MEN) type 1 and 2, von-Hippel-Lindau disease, tuberous sclerosis, and neurofibromatosis [12, 13, 18].

Patients with NE tumours may or may not have symptoms attributable to hormonal secretion. These hormones can cause debilitating symptoms such as flushing, diarrhea, and congestive heart failure in patients with carcinoid syndrome, hypertension in patients with pheochromocytoma, and symptoms attributable to secretion of insulin, glucagon, gastrin, and other peptides in patients with pancreatic NE tumours [29, 30]. While NETs are relatively rare, the estimated incidence of NETs in the United States is increasing since the 1970s according to the SEER database [23].

Currently, the preferred treatment for patients with local or regional disease is surgical resection and prognosis following complete surgical resection is quite favorable [72]. However, NETs often metastasize to the liver before they are diagnosed making curative resection unlikely. Despite the tumour's slow growth and indolent nature, current chemotherapeutic options have not altered survival [82]. While somatostatin analogues have improved the control of carcinoid

syndrome, their effects on tumour growth are limited [85]. Therefore, it is imperative that new therapies targeting the signaling pathways involved in NE tumours are developed.

NETs generally do not have aberrant expression of common tumour suppressors or oncogenes [19], hence therapeutic options have focused on growth factor receptors such as the vascular endothelial growth factor receptors (VEGFRs) and platelet-derived growth factor receptors (PDGFRs). Previous studies have demonstrated the role of VEGFRs in correlating with tumour metastasis, but little is known about PDGFRs [103]. Platelet derived growth factor (PDGF) is a family of peptides that exert their cellular effects by activating two structurally related cell-surface receptor tyrosine kinases (PDGFR-α and -β). Aberrant expression of PDGF and activation of PDGFRs was implicated in the development of aggressive carcinomas with lymphatic and distant metastases [104]. NETs also express PDGF and PDGFRs. In fact, expression of PDGF has been found in 70% and 60% of carcinoid and pancreatic NE tumours, respectively. PDGFR-α has been found on both tumour cell and its surrounding stroma, while PDGFR-β was only found in the stroma [99, 100].

Activating mutations of PDGFR and autocrine PDGF signaling are common in tumour progression. For example, PDGFR-α and PDGF ligands are frequently over-expressed in glioblastoma multiforme (GBM). In fact, forced expression of PDGFR-α and its ligand confers growth advantages in both human and mice glioma cells [105]. Similarly, autocrine PDGF signaling is also involved in many soft tissue sarcomas. In addition, autocrine PDGF signaling has also been implicated in human breast carcinoma during TGF-β induced epithelial-mesenchymal transition [106]. More recently, it has been shown in our laboratory that the

expression of PDGFR-α isoform is associated with increased invasive potential in papillary thyroid cancers [107]. All these findings suggest an important role of PDGF signaling pathway in "driving" the development of aggressive tumours across different types of human carcinomas. The purpose of our study was to assess the role of PDGFR-α in human neuroendocrine tumours.

2.2. Patients and methods

2.2.1. Patient specimens

Ethics approval was obtained through the University of Alberta Heath Research Ethics Board ID Pro00018758. Specimens prepared for primary cell culture or tissue banking were placed in culture media or OCT (Optimal Cutting Temperature compound), respectively, within 10 minutes of devascularisation.

2.2.2. Reagents and Antibodies

The following antibodies were used for immunoblotting: phospho-Erk1/2(Thr 202/Tyr204) (E10: #9106), Akt (#9272), phospho-Akt (Ser473) (587F11: #4051), PDGFR-α (D13C6: #5241), phospho-PDGFR-α/β (Tyr849)/(Tyr857) (C43E9: #3170), were all from Cell Signaling Technology (Danvers, MA, USA). The PDGFR-β (11H4: sc-80991), Pax-8 (PAX8R1: sc-81353), TTF-1(8G7G3/1: sc-53136) and total Erk1 antibody (K-23: sc-94) were from Santa Cruz Biotechnology, (Dallas, TX, USA). Anti-γ-tubulin antibody (GTU-88: T6557) was from Sigma Aldrich (St. Louis, MO, USA). For cell sorting of PDGFR-α positive and negative cells,

PDGFR-α / PE conjugate (D13C6: #8533) from Cell Signaling Technology (Danvers, MA, USA) was used. PDGF-BB was purchased from GIBCO (Carlsbad, CA, USA). Crenolanib, Everolimus, and Sunitinib malate, were purchased from Selleckchem (Houston, TX, USA).

2.2.3. Cell culture

BON-1 experimental cell line was generously provided by Dr. S. Ezzat, University of Toronto, Canada. Primary cell culture and experimental cell lines were maintained in DMEM/ F12 media supplemented with 10% FBS and 5H (Insulin at 10μg/ml, Hydrocortisone at 10nM, Transferrin at 5μg/ml, Glcyl-histyl, lysine acetate at 10ng/ml and Somatostatin at 10ng/ml) at 37^{o}C. QGP-1 experimental cell line was generously provided by Japan Health Science Foundation and was maintained in RPMI with 10% FBS.

2.2.4. Isolation of primary neuroendocrine cells

Primary neuroendocrine cancer cells were obtained from using the Cancer cell Isolation Kit (Panomics, Inc. Fremont, CA, USA). Tissue was minced to small pieces under aseptic conditions and digested for 2 hours with gentle mixing at 37°C. Cancer cells were subsequently purified following manufacturer's protocol. Isolated primary cancer cells were cultured in DMEM/F12 medium supplemented with 10% FBS and 5H at 37^{o}C.

2.2.5. Western blot analyses

Cells were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, 2 mM pepstatin, and 1:100 protease inhibitor cocktail set III on ice. After centrifugation at 4°C at 132,000 rpm for 10 minutes, the supernatant was harvested as the total cellular protein extract, aliquoted and stored at –80°C. The protein concentration was determined using Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Running samples were prepared by adding a sample-reducing agent and SDS sample buffer, incubating at 98°C for 5 minutes. Aliquots (usually 50 µg) of protein extract samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. Membranes were blocked with 5% non-fat milk in TBS containing 0.05% Tween-20 for 60 minutes, followed by incubation with primary antibodies at 4°C overnight. Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (Pierce Biotechnology, Rockford, IL, USA) and visualized with SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, Rockford, IL, USA).

2.2.6. qRT-PCR

Total RNA was extracted from cell cultures using Trizol reagent as per manufacturer's protocol and cDNA was generated with a high-capacity cDNA reverse transcription kit (Invitrogen, Burlington, ON, Canada), according to the manufacturer’s protocol. Amplification of various

transcripts was performed using the following primers - Chromogranin: 5'-TCGTTGAGGTCATCTCCGAC-3' (forward), 5'-AGGATCCGTTCATCTCCTCG-3' (reverse), Norepinephrine re-uptake transporter: 5'-TCCTCCTGTTCGTGGTTGTG -3' (forward), 5'-CCTGCGTGCTGAGGAACTTA-3' (reverse), Synaptophysin: 5'-CTGTGACCTCGGGACTCAAC-3' (forward), 5'-AGACTGGGCACCTAGTGGAT-3' (reverse), PDGFR-α: : 5'-TAGTGCTTGGTCGGGTCTTG-3' (forward), 5'-TTCATGACAGGTTGGGACCG-3' (reverse), PDGFR-β: : 5'-GCTCACACTGACCAACCTCA-3' (forward), 5'-GGTGGGATCTGGCACAAAGA-3' (reverse), GAPDH: : 5'-GTCTCCTCTGACTTCAACAGCG-3' (forward), 5'-ACCACCCTGTTGCTGTAGCCAA-3' (reverse).

2.2.7. Gene quantitation with TaqMan arrays

A customized 384-well TaqMan array was used for the detection of user-specific gene targets. Relative levels of gene expression were determined from the fluorescence data generated during PCR using the ABI PRISM®7900HT sequence detection system and relative quantitation software. The TaqMan Array is designed for two-step RT-PCR. In the reverse-transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from the High Capacity cDNA Archive Kit. In the reverse-transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from the High Capacity cDNA Archive Kit. Genes induced >2.0-fold or repressed less than 0.5-fold versus baseline have been considered as significantly regulated and included into interpretation. All experiments were performed with 3 replicates.

2.2.8. Small interfering RNA (siRNA) transfection

RNA interference (RNAi) is a recently developed method of gene silencing that introduces double stranded RNA (dsRNA) into cells resulting in post-transcriptional gene silencing. The small interfering RNA (siRNA) is a 21-23 nucleotide (nt) double stranded RNA with 2 nt overhangs at each 3' ends. Upon transfection into mammalian cells, they are incorporated into an RNA-induced silencing complex (RISC), a complex that cleaves mRNA complementary to the siRNA leading to silencing of that gene. To selectively knock down the expression of the PDGFR-α receptor in the primary neuroendocrine cell culture, transient transfection of siRNA against PDGFR-α receptor (ON-TARGET plus, Thermo Scientific, Rockford, IL, USA) was carried out using the X-fect^{TM} transfection reagent as recommended by the manufacturer (Clontech Laboratories, Inc., Mountain View, CA, USA). On-Target sequences are as follows: 5'-CGAGACUCCUGUAACCUUA-3', 5'-GAGCUUCACCUAUCAAGUU-3', 5'-GACAGUGGCCAUUAUACUA-3', 5'-GAAUAGGGAUAGCUUCCUG-3'. Scrambled sequence of the siRNA target sequence was used as negative control (Fig. 2.1.).

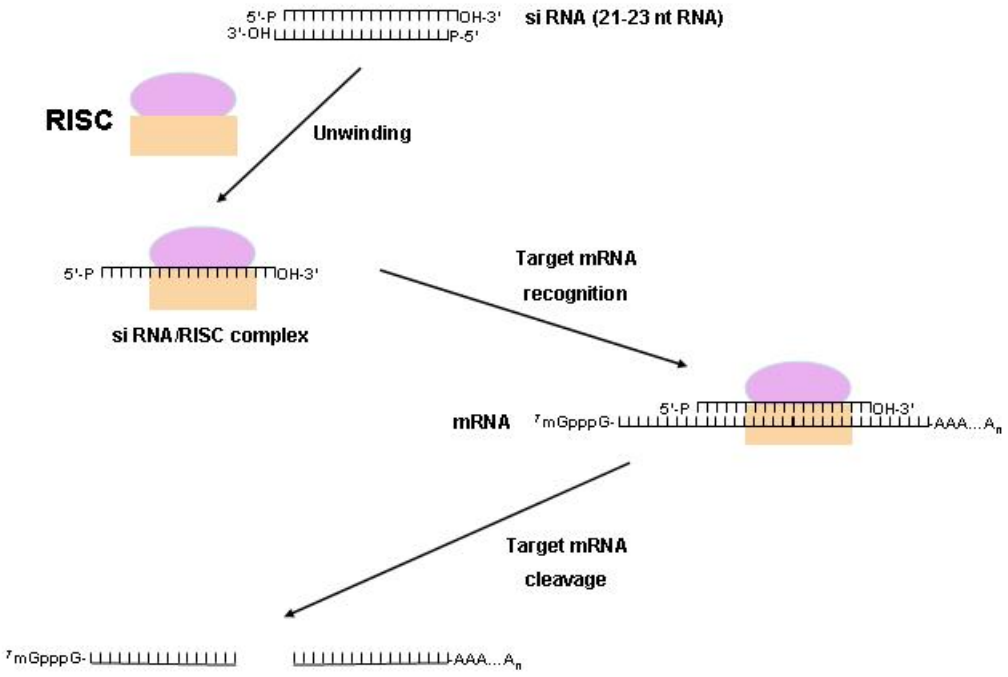


Figure 2.1. Model of siRNA gene silencing mechanism. Small interfering RNAs (siRNAs) are transfected into cells, where they are assembled into an RNA-induced silencing complex (RISC). In the RISC, siRNAs are unwound by the helicase activity of the complex and target complimentary mRNA, through base pairing, for cleavage and degradation by the RISC complex Reproduced from *Small RNA: can RNA interference be exploited for therapy?* Figure from [108].

2.2.9. Gene transfer

To express human PDGFR-α complementary DNA, a doxycycline-inducible retrovirus system was used (Lenti-X Lentiviral Expression Systems; Clontech Laboratories, Inc., Mountain View, CA, USA). BON-1 cells were first transduced with the LVX-Tet-On advanced lentivirus (Neo+) followed by selection in G418 (1.0 mg/mL). Resistant cells were then transduced with the LVX-Tight-Puro (Puro+) vector or sequence-verified derivatives expressing wild-type human PDGFR-

α complementary DNA, followed by selection in puromycin (2.5 μg/mL). Complementary DNA expression was induced by addition of doxycycline (2 μg/mL).

2.2.10. Migration assay

A monolayer scratch assay was used to compare the migratory ability of PDGFR-α expressing cell lines compared to cells transfected with empty vector. Both primary neuroendocrine cell cultures and experimental cell lines (BON-1 and QGP-1) were cultured to 80-90% confluence. Culture media were supplied with PDGF-BB at a concentration of 50ng/ml. A mechanical wound was created by manually scratching the cell monolayer with a p1000 or p200 pipet tip. Cellular debris was removed by washing the monolayer with PBS. Images were photographed using phase contrast microscopy at 0, 24, 48, 72, and 96hours. The minimum distance in micrometers between the wound edges of the scratch area was analyzed using Adobe Photoshop 7.0. Migration assays utilizing primary cell cultures were performed with 6 replicates, whereas those involving the transfected experimental cell lines were performed in replicates of 4. Mitomycin C (0.25μg/ml) was also used to limit cellular proliferation.

2.2.11. Cell proliferation assay

Cells were plated in 96-well plates (10,000 cells each well) in 100ul of media. The media from all groups were supplemented with doxycycline (2ug/ml) and PDGF-BB (50ng/ml). At 24, 48, 72, and 96hr, cell proliferation was measured with MTS assay according to the manufacturer's protocol. The cell proliferation assay was performed with eight replicates.

2.2.12. Colony formation assay

Adherent colony formation assays were performed as previously described. Serial dilution was carried out to achieve a single-well density of approximately 100 cells/well using a six-well plate. Cells were cultured in 10% FBS supplemented growth medium with PDGF-BB (50ng/ml) and allowed to form colonies for 20 days. Colonies were stained with 0.5% crystal violet solution in 25% methanol and counted.

2.2.13. Mouse xenograft model

All experiments were conducted in accordance with the guidelines of the University of Alberta Animal Care and Use Committee (ACUC) and the Canadian Council of Animal Care. Fox Chase Beige SCID male mice, 8-10 weeks old, were purchased from Charles River Laboratories, and allowed to acclimate for 7 days. Cells were maintained in DMEM media supplemented with 10% fetal bovine serum plus 5H prior to inoculation. BON-1 and QGP-1 cells (1.5×10^6) expressing either PDGFR-α protein or empty vector were inoculated subcutaneously (1:1 v/v matrigel–PBS) on the flanks of SCID mice that had received a 0.5 mg slow-release doxycycline pellet subcutaneously 48 hours prior to cell inoculation. Tumour growth was followed and documented and animals were sacrificed once the tumours reached a $1cm^3$ size.

2.2.14. Statistical analysis

Data were expressed as the mean ± standard error of mean from a minimum of three independent experiments. Statistical analyses were performed using the two-tailed Student's *t* test for unpaired samples, with equal variance. Statistical tests are two-tailed with a P value <0.05 considered to be statistically significant. The SAS computer program SAS (r) 9.2 (TS1M0) was used to perform the analysis.

2.3. Results

2.3.1. Expression of PDGFR-α in clinical NET specimens

Of the 16 Tumour Bank NET samples, PDGFR-α expression was evident in 81% (n = 13/16) which is in agreement with previous study by Chaudhry et al [100] confirming the expression of PDGFR-α in NETs (Fig. 2.2.). Given the clinical significance of PDGFR-α expression in correlation to metastatic potential in other malignancies such as glioma and papillary thyroid cancers, it would be of clinical interest to see if the expression of PDGFR-α would indeed confer metastatic advantages in NETs. RT-PCR was also performed for each of the 16 samples to confirm their neuroendocrine origin by specifically looking at expression of chromogranin A and synaptophysin (Fig. 2.3.). All 16 samples had chromogranin A expression and 12 out of 16 samples also expressed synaptophysin. As a control for staining by western blot, T47D cell line (no native PDGFR-α expression in parental cell line) transduced with PDGFR-α and empty-vector was used as positive and negative control for PDGFR-α expression, respectively.

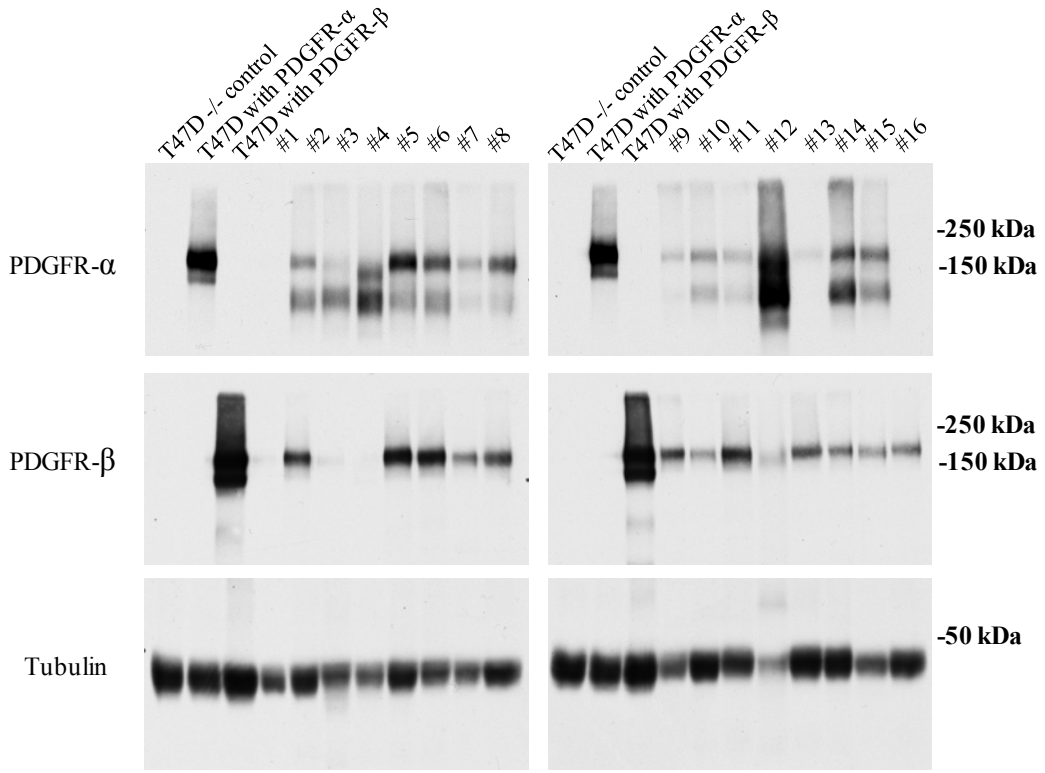


Figure 2.2. Western blots of PDGFR-α in patient specimens, where the majority of the specimens were obtained from metastatic lesions. T47D cell line was used as an internal control.

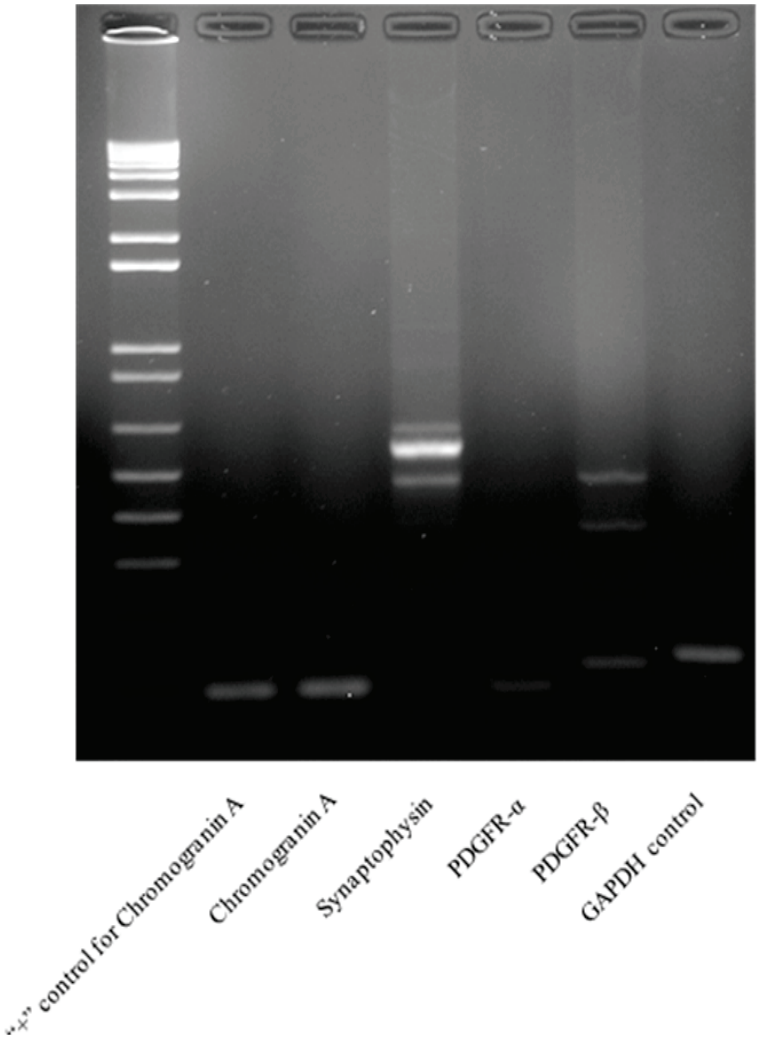
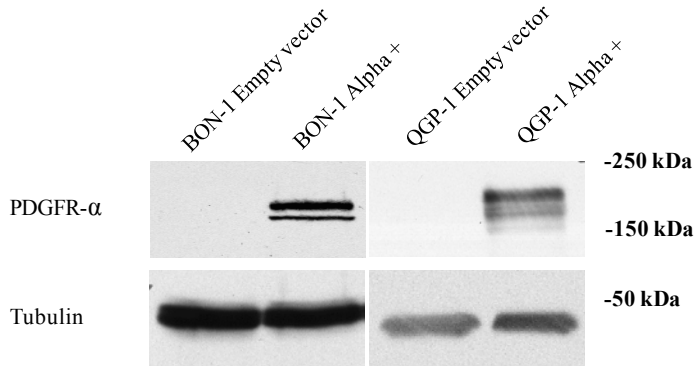


Figure 2.3. Representative PCR gel of patient specimen demonstrating expression of neuroendocrine-specific biomarkers such as the Chromogranin A and Synaptophysin.

2.3.2. Expression of PDGFR-α in BON-1 and QGP-1 experimental cell lines

The finding of PDGFR-α in clinical NET specimens prompted us to assess the expression of PDGFR-α in the BON-1 and QGP-1 cell lines. The expression of PDGFR-α was only detected at the mRNA level as indicated by RT-PCR, but not at the protein level as shown in western blot (Fig. 2.4. a and b). In fact, a comprehensive search across multiple cancer cell lines revealed that pancreatic cancer lines (including both neuroendocrine and non-neuroendocrine cancer cell lines) have very low level of PDGFR-α mRNA expression in general (Fig. 2.5.). Hence, stable BON-1 and QGP-1 constructs with inducible PDGFR-α expression were established to characterize the role in PDGFR-α in promoting tumour metastasis in NETs given the scarcity of other well-established neuroendocrine cell lines.

a)



b)

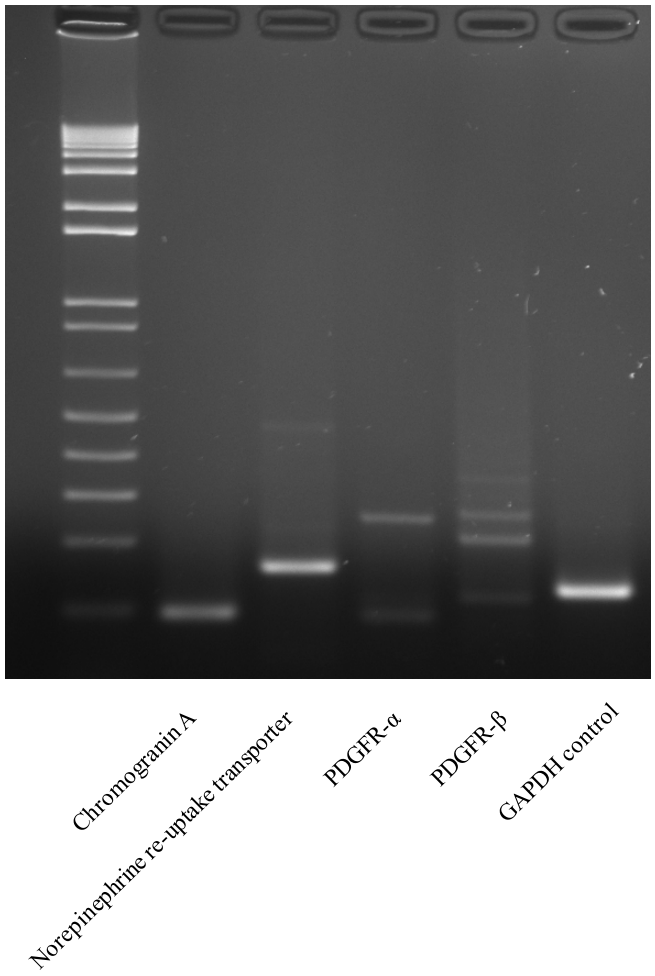


Figure 2.4. a) Western blots illustrating the lack of native PDGFR-α protein expression in both BON-1 and QGP-1. **b)** Representative PCR gel showing the presence of PDGFR-α mRNA expression in QGP-1 (Identical profile for BON-1, results not shown).

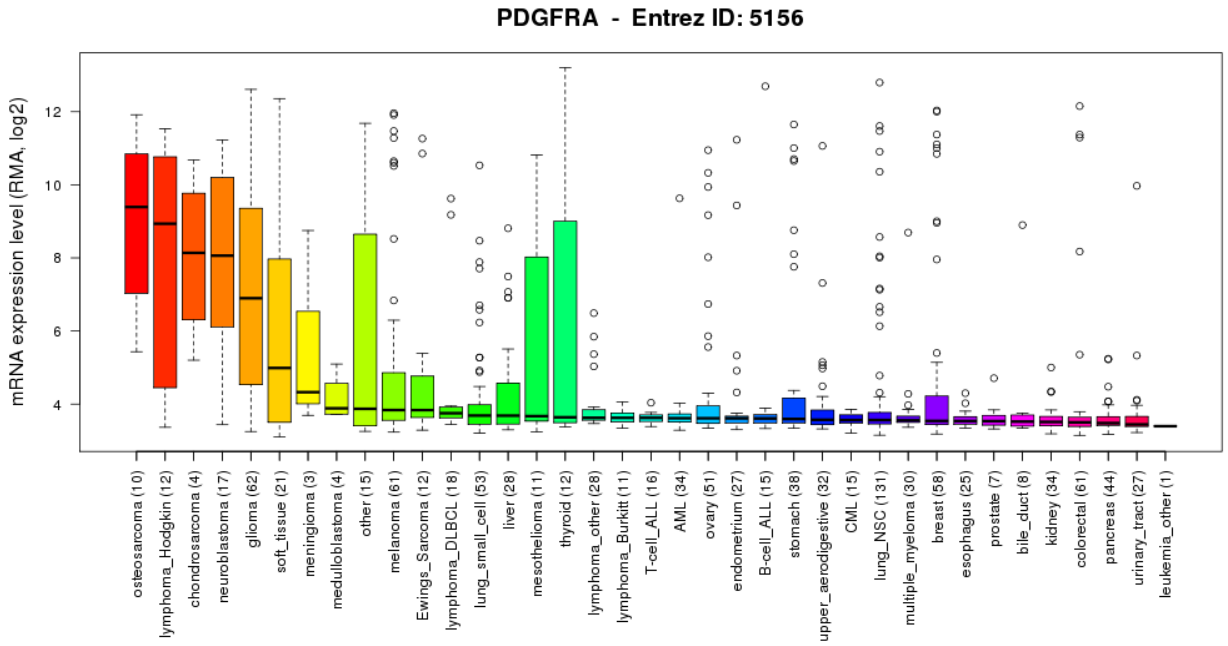


Figure 2.5. PDGFR-α mRNA levels in different cancer cell lines as retrieved from the Cancer Cell Line Encyclopedia from the Broad Institute, ID # 5156 [109]. At the time of publication, the Cancer Cell Line Encyclopedia contained gene expression data for 1046 cancer cell lines. The number of cell lines per cancer type assayed is indicated in the x-axis. RMA, robust multi-array average (y-axis); AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; DLBCL, diffuse large B-cell lymphoma; NSC, non-small cell.

2.3.3. Phenotypic characterization of PDGFR-α expressing BON-1 and QGP-1

a) Proliferation assay

The presence of PDGFR-α did not alter the proliferative capacity of both BON-1 and QGP-1 cells. The BON-1 demonstrated a pattern of linear growth across all time points, whereas the QGP-1 appeared to reach a growth-plateau at around 48hr time point (Fig. 2.6. and 2.7.). There were no statistically significant differences between the PDGFR-α expressing cells compared to the empty vector controls.

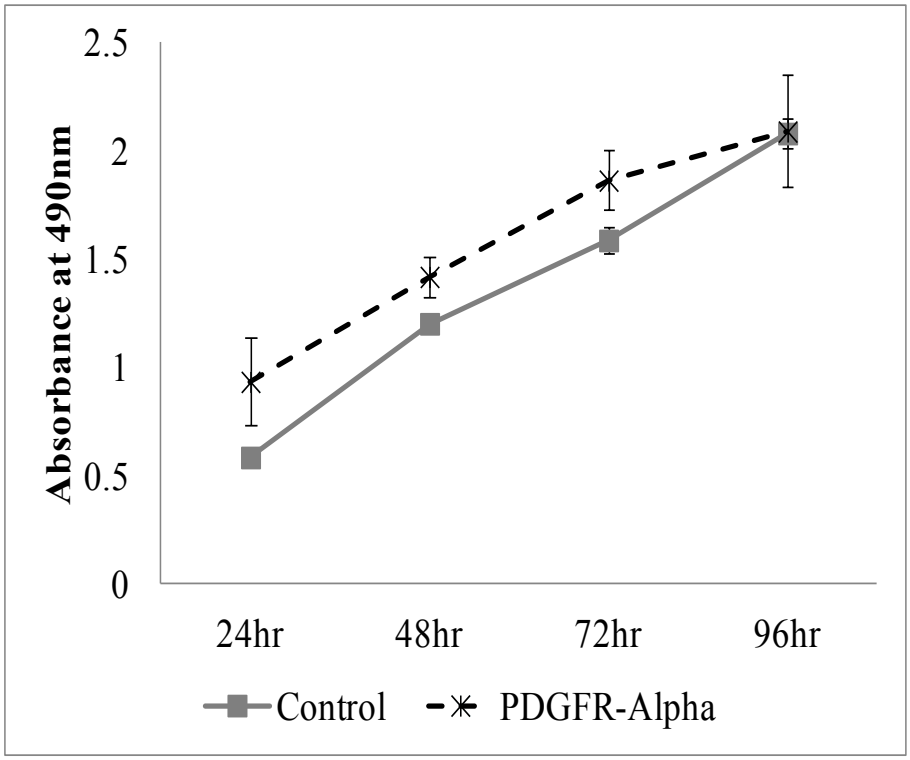


Figure 2.6. Proliferation assay of PDGFR-α expressing BON-1 cells. The absorbance value measured at 490nm was used as a marker for cellular proliferation in the MTS assay. The rate of proliferation of PDGFR-α expressing BON-1 cells was plotted against the empty vector control cells at various time points.

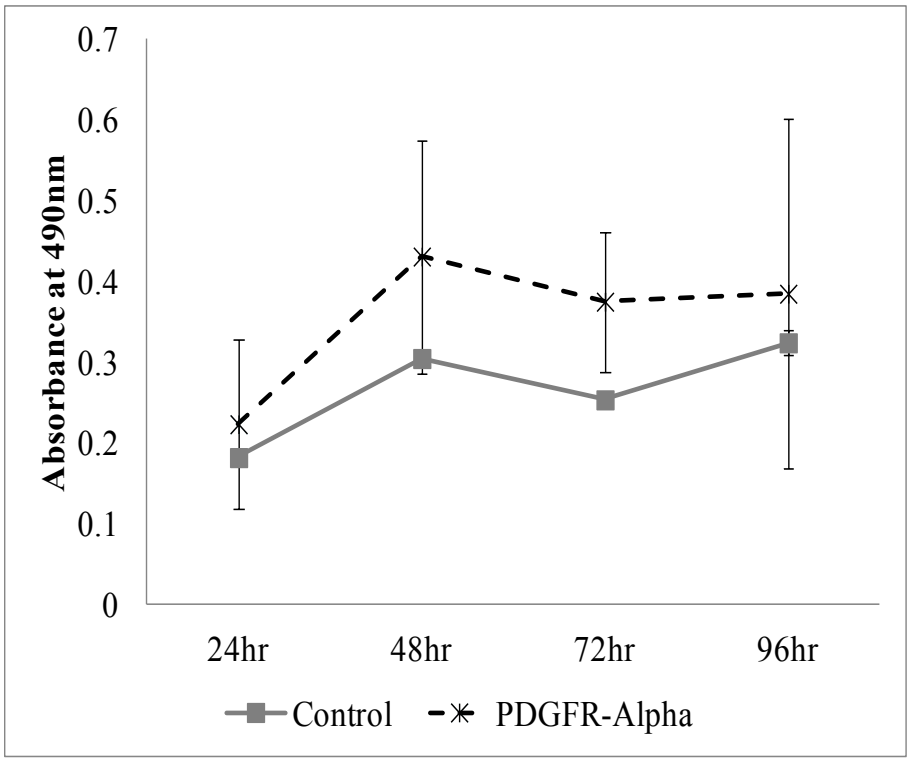


Figure 2.7. Proliferation assay of PDGFR-α expressing QGP-1 cells. The absorbance value measured at 490nm was used as a marker for cellular proliferation in the MTS assay. The rate of proliferation of PDGFR-α expressing QGP-1 cells was plotted against the empty vector control cells at various time points.

b) Colony formation assay

The presence of PDGFR-α significantly enhanced the colony-formation capability of both BON-1 and QGP-1 (Fig. 2.8.). BON-1 cells with PDGFR-α expression formed significantly larger and more colonies than the empty-vector control (Fig. 2.9. and 2.10.). A similar trend was also observed in QGP-1 (Fig. 2.11. and 2.12.).

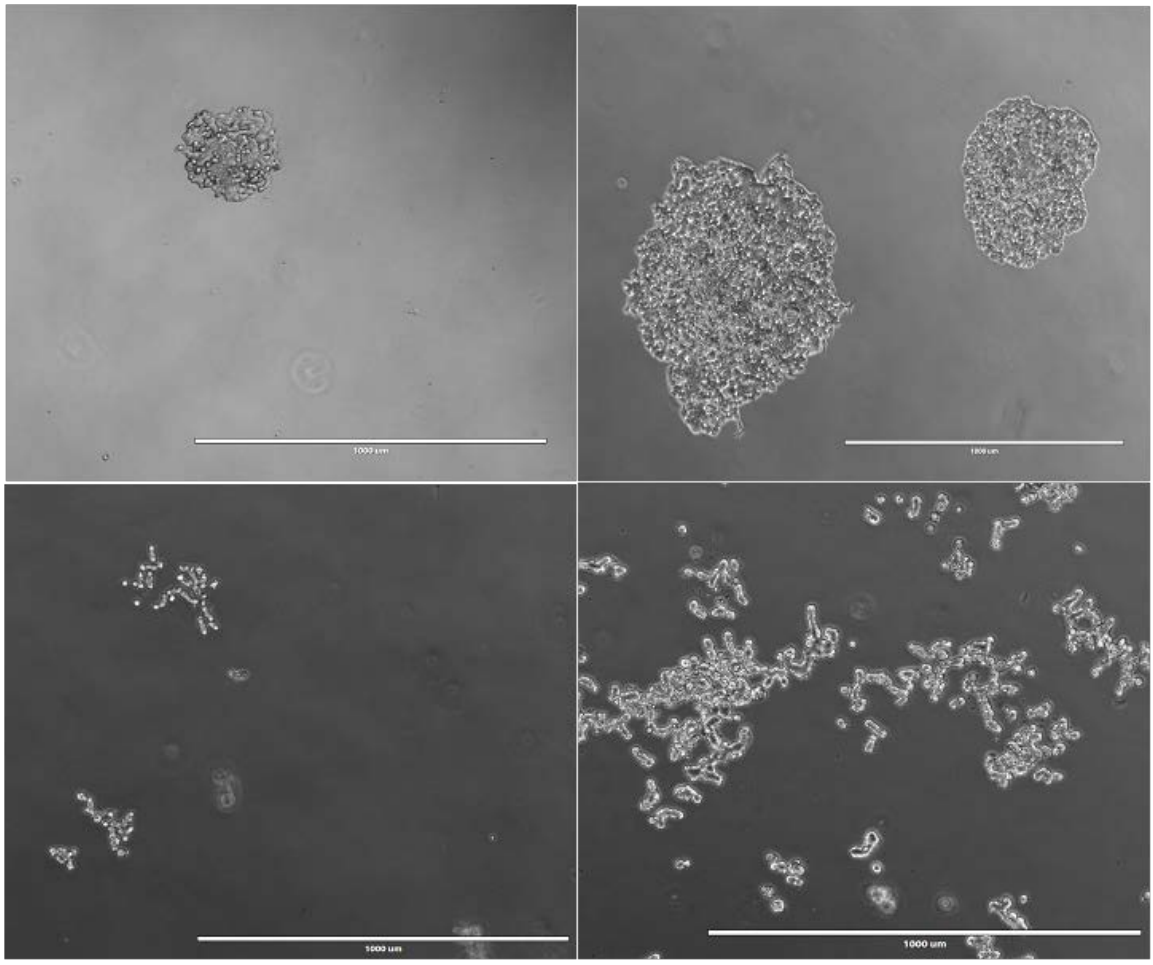


Figure 2.8. Representative microscopy images of cell colonies at 4X magnification. Top left (Empty vector BON-1), Top right (PDGFR-α expressing BON-1), Bottom left (Empty vector QGP-1), Top right (PDGFR-α expressing QGP-1)

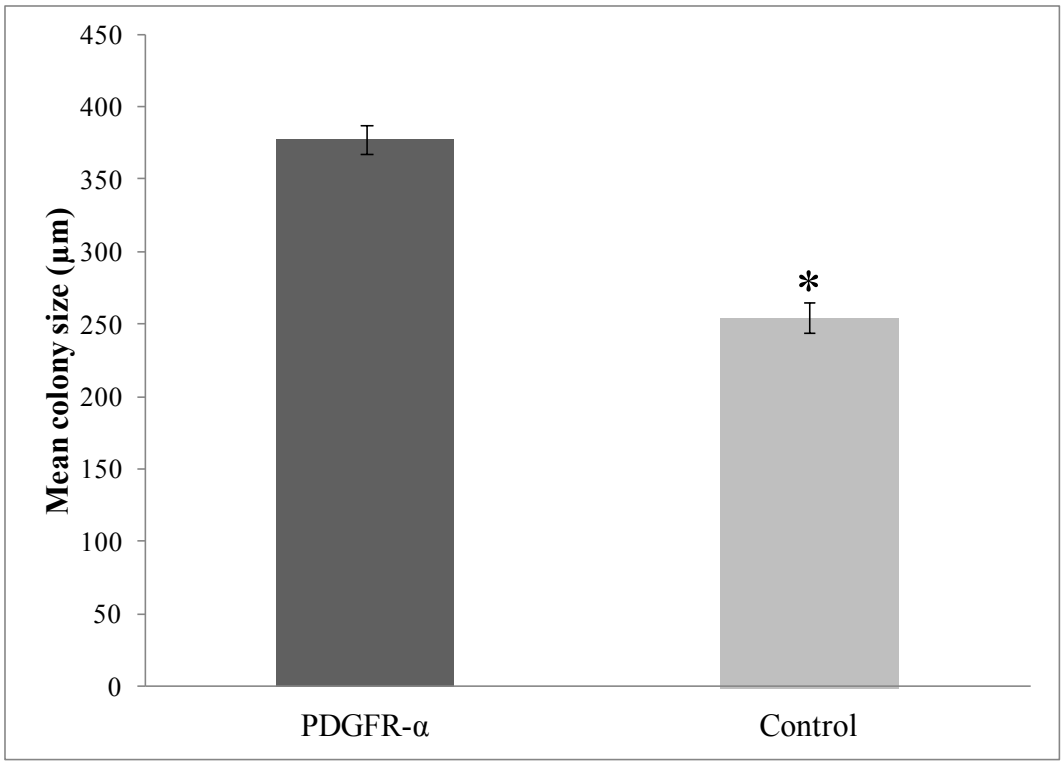


Figure 2.9. Mean 20-day colony size (largest cross-sectional diameter measured in µm) comparing PDGFR-α expressing BON-1 cells to empty vector control cells. * = $p < 0.05$

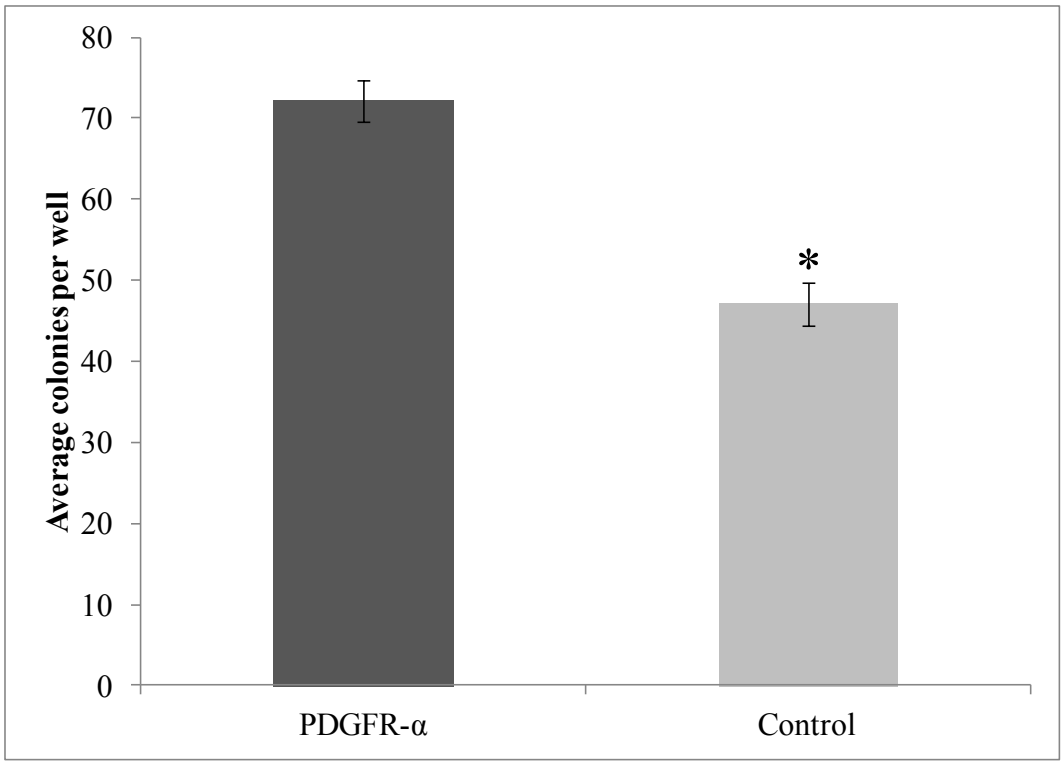


Figure 2.10. Average number of colonies per well comparing PDGFR-α expressing BON-1 cells to empty vector control cells. * = $p < 0.05$

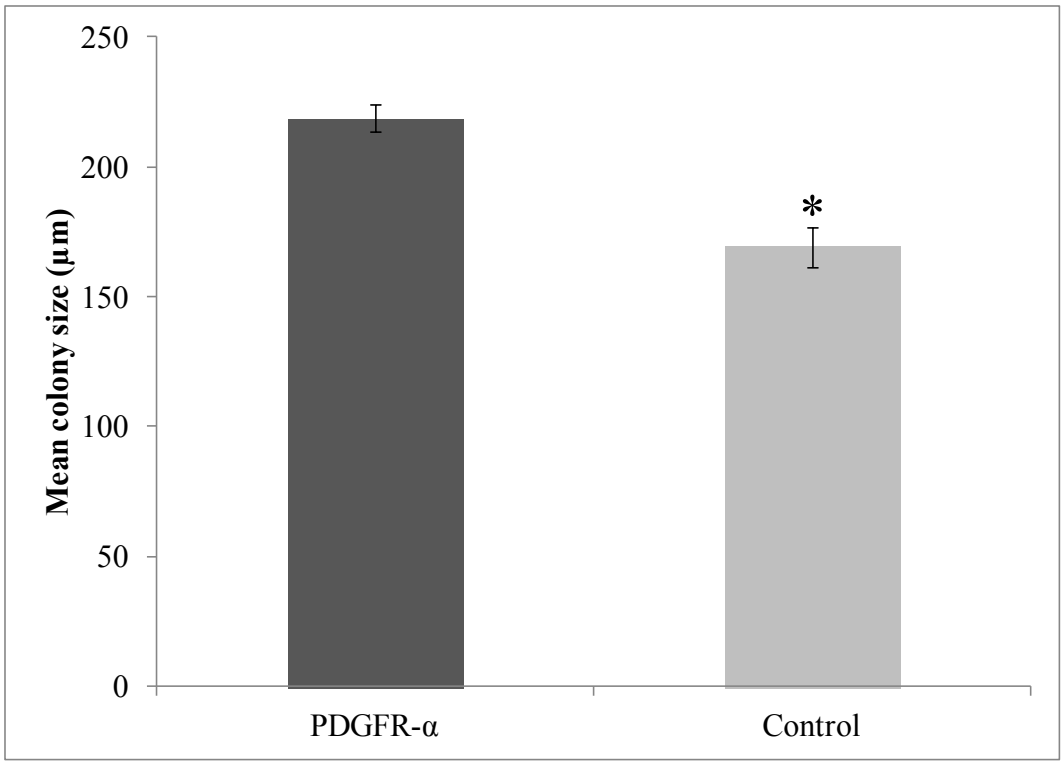


Figure 2.11. Mean 20-day colony size (largest cross-sectional diameter measured in µm) comparing PDGFR-α expressing QGP-1 cells to empty vector control cells. * = $p < 0.05$

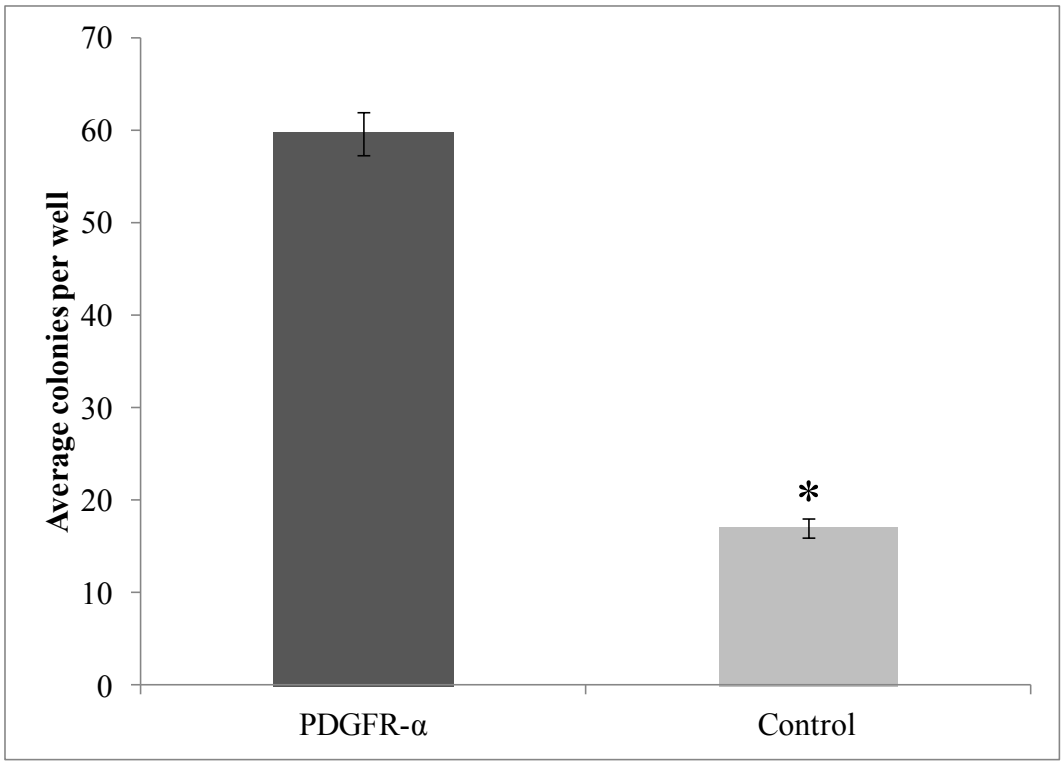


Figure 2.12. Average number of colonies per well comparing PDGFR-α expressing QGP-1 cells to empty vector control cells. * = $p < 0.05$

c) Migration/ Wound healing assay

PDGFR-α expressing BON-1 cells migrated significantly faster than the empty vector control cells and such migratory advantage amplified with time (Fig. 2.13.). Since the expression of PDGFR-α did not confer any proliferative advantage, it was evident that this observed increase in migration / invasion was not caused by enhanced proliferation. In addition, mitomycin C (at a non-toxic dose of 0.25μg/ml) was also used as a control for cellular proliferation. In contrast, PDGFR-α expressing QGP-1 cells did not migrate significantly faster than the empty-vector control despite demonstrating a trend in favour of QGP-1 with the expression of PDGFR-α; however, the differences in their migratory potential did not reach statistical significance (Fig. 2.14.).

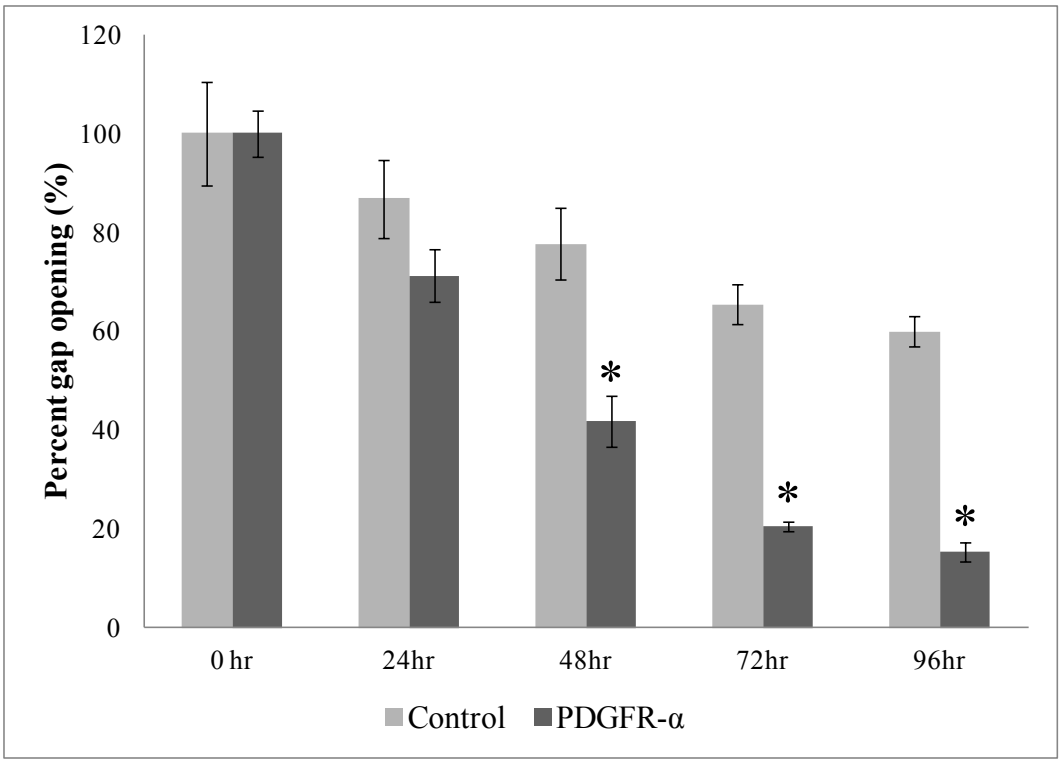


Figure 2.13. Migration assay of PDGFR-α expressing BON-1 cells. BON-1 migration is enhanced in the presence of PDGFR-α expression. BON-1 cells with stable PDGFR-α expression has increased migration at 48, 72, and 96hr time points. PDGF-BB (50ng/ml) was added to the culture media. * = $p < 0.05$

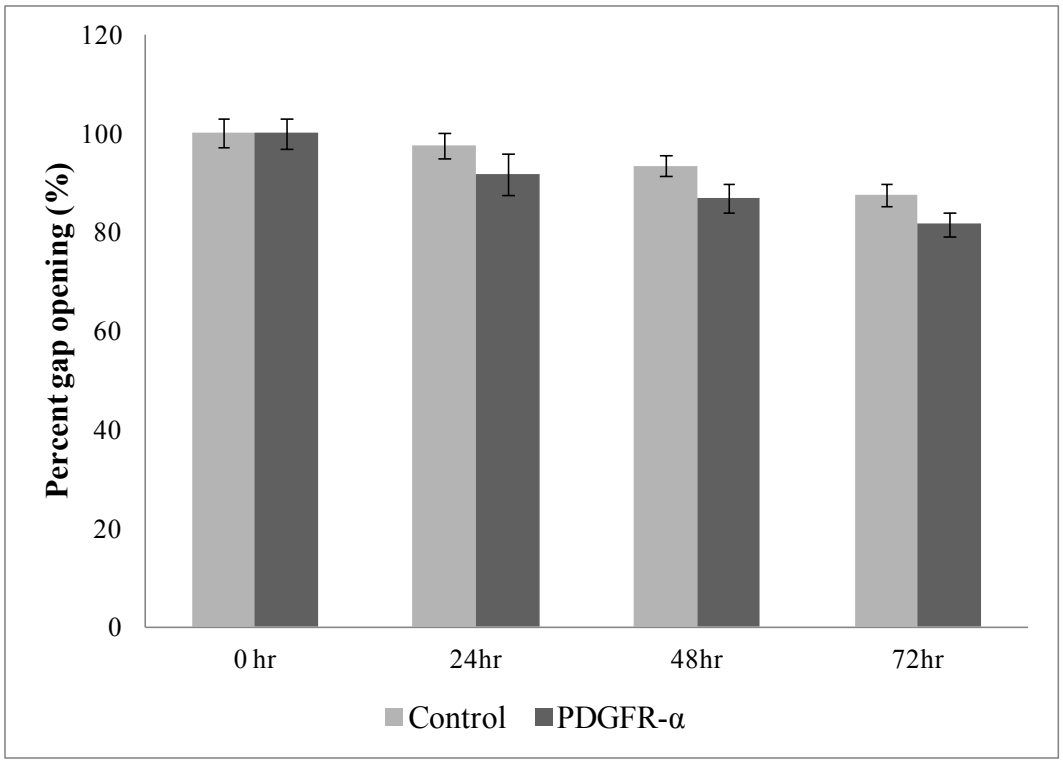


Figure 2.14. Migration assay of PDGFR-α expressing QGP-1 cells. QGP-1 migration is not enhanced in the presence of PDGFR-α expression. QGP-1 cells with stable PDGFR-α expression appears to migrate at a faster rate at both 48hr and 72hr timepoints. However, the differences do not reach statistical significance.

2.3.4. PDGFR-α activation increases migratory potential in BON-1 and it can be reversed by selective PDGFR-α blockade utilizing either tyrosine kinase inhibitor or siRNA

Having demonstrated the presence of PDGFR-α in clinical specimens, we were able to show that the migratory phenotype appears to link to the presence of PDGFR-α expression in both the BON-1and QGP-1 cell lines. The presence of PDGFR-α significantly enhanced the migratory potential of BON-1. With the addition of crenolanib, a selective PDGFR-α inhibitor, we were able to abate the phenotype (Fig. 2.15.). Western blot analysis also confirmed the reduction in p-PDGFR-α activation as a result of PDGFR-α inhibition (Fig. 2.16.)

To further demonstrate that the PDGFR-α subunit is crucial in mediating increased invasive potential in NETs, we used siRNA to selectively disrupt expression of PDGFR-α in primary cell cultures generated from clinical specimens (n = 3) exhibiting PDGFR-α and examined the subsequent effect on metastatic potential using migration assay. It was evident that the rate of migration of the primary cell culture was significantly reduced by the disruption of PDGFR-α expression (Fig. 2.17.). All three primary cultures were tested for their neuroendocrine origin using RT-PCR before and after the experiment. Western blots for both PDGFR-α and PDGFR-β were performed to quantify the percentage knock-down as well as to rule out non-specific targeting of PDGFR- β by siRNA treatment, respectively (Fig. 2.18.).

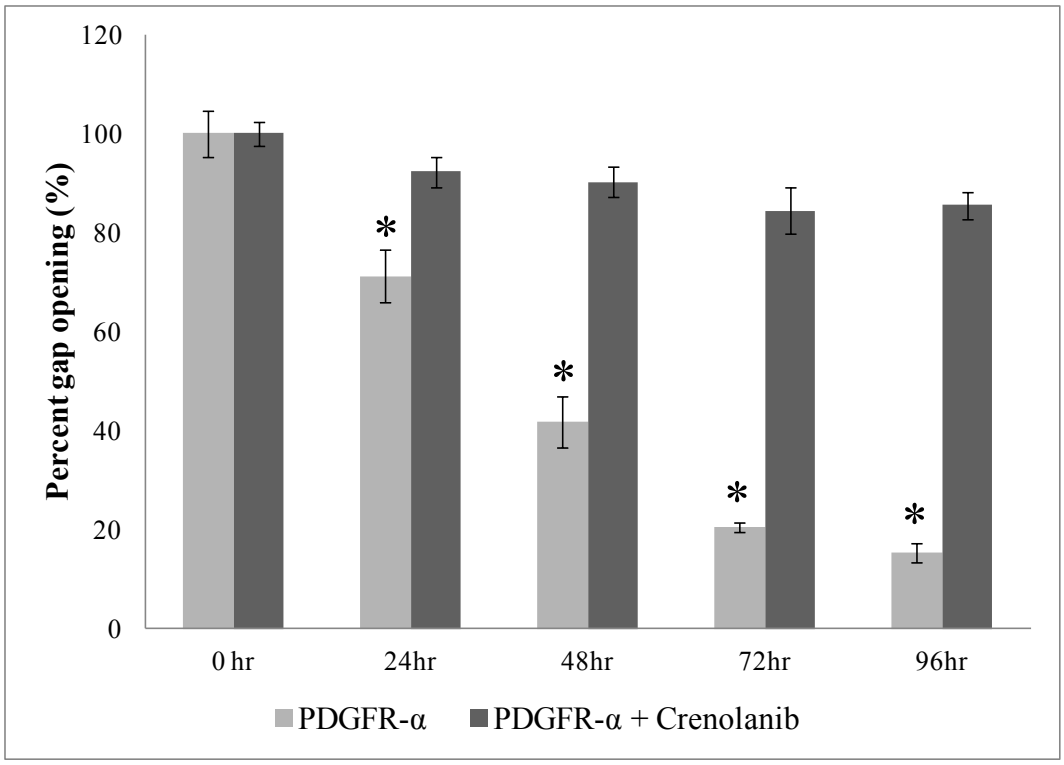


Figure 2.15. Inhibition of enhanced BON-1 migration by selective PDGFR-α inhibitor (crenolanib). The increased migratory potential observed in PDGFR-α expressing BON-1 cells was reversed by crenolanib treatment. PDGF-BB (50ng/ml) was added to the culture media. * = $p < 0.05$

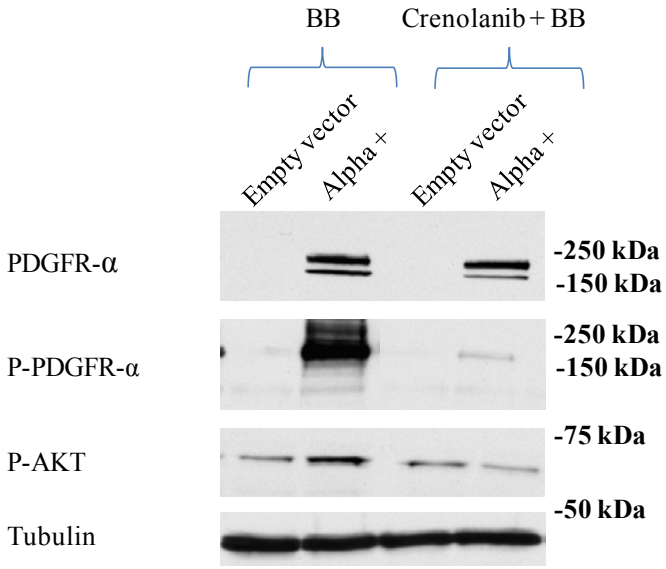


Figure 2.16. Western blots for BON-1 treated with overnight PDGF-BB (50ng/ml) and crenolanib (2μM) looking specifically at p-PDGFR-α and p-AKT

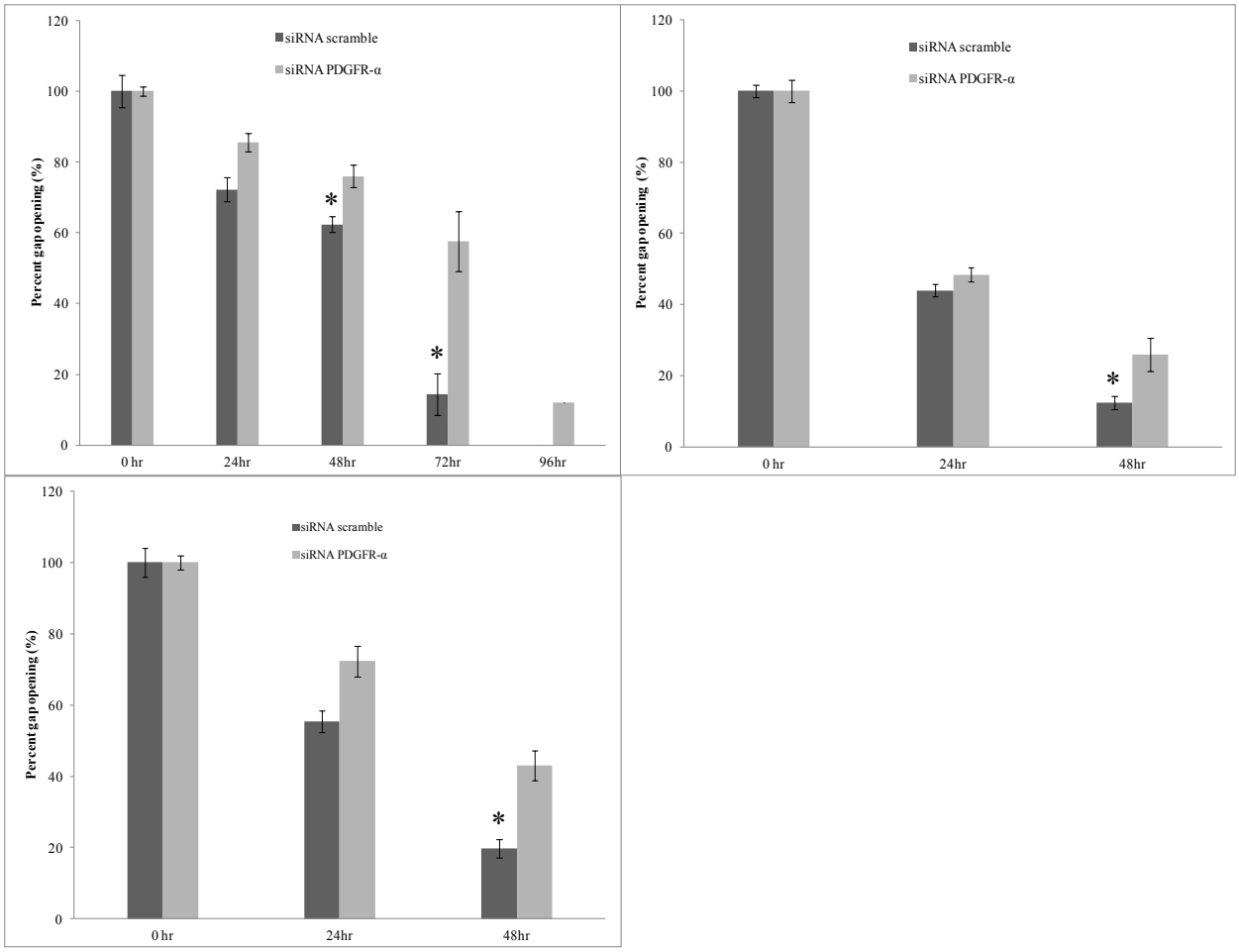


Figure 2.17. Migration assay of primary cell culture generated from clinical specimens. The rates of migration of primary NET cultures were significantly reduced with the disruption of PDGFR-α expression via SiRNA. * = $p < 0.05$

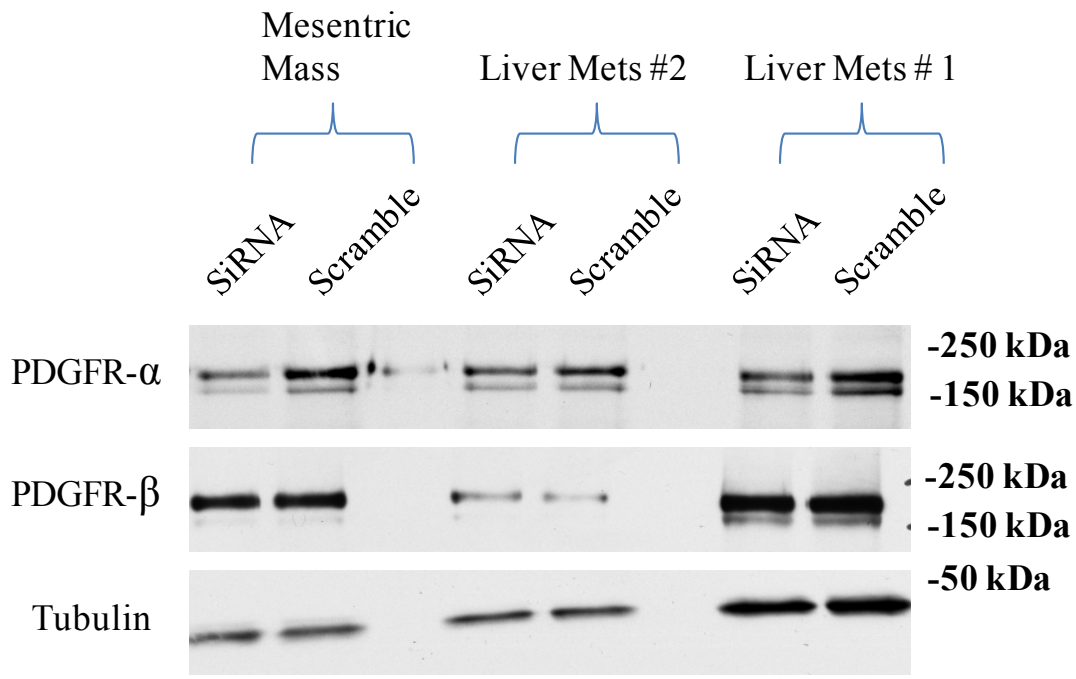


Figure 2.18. Western blots of PDGFR-α and PDGFR-β for primary cell cultures generated from clinical specimens. Densitometry revealed 67%, 26%, and 48% knock down in PDGFR-α expression in mesenteric mass, liver mets #2, and liver mets #1, respectively. PDGFR-β expression, in contrast, remained unaltered.

2.3.5. PDGFR-α activation in BON-1 and QGP-1 respectively promotes tumour angiogenesis and growth *in vivo*

The presence of PDGFR-α in BON-1 did not enhance its tumourigenicity *in vivo* (Fig. 2.19.). There were no differences in tumour weight between the PDGFR-α expressing BON-1 and the control. On the other hand, PDGFR-α expression in QGP cells promoted tumour growth. The average weight of PDGFR-α expressing xenografts was approximately 2.5 times heavier than their paired controls (Fig.2.20.). In addition, the PDGFR-α bearing xenografts appeared to be more vascular in nature upon gross inspection. Given that NETs are vascular tumours often associated with tissue desmoplastic reaction, micro-vascular density was evaluated by immune labeling with the anti-cluster of differentiation 31 antibody. In line with our observation, both PDGFR-α expressing BON-1 and QGP-1 have a significantly higher vascular density than the control (Fig.2.21. , 2.22. a and b).

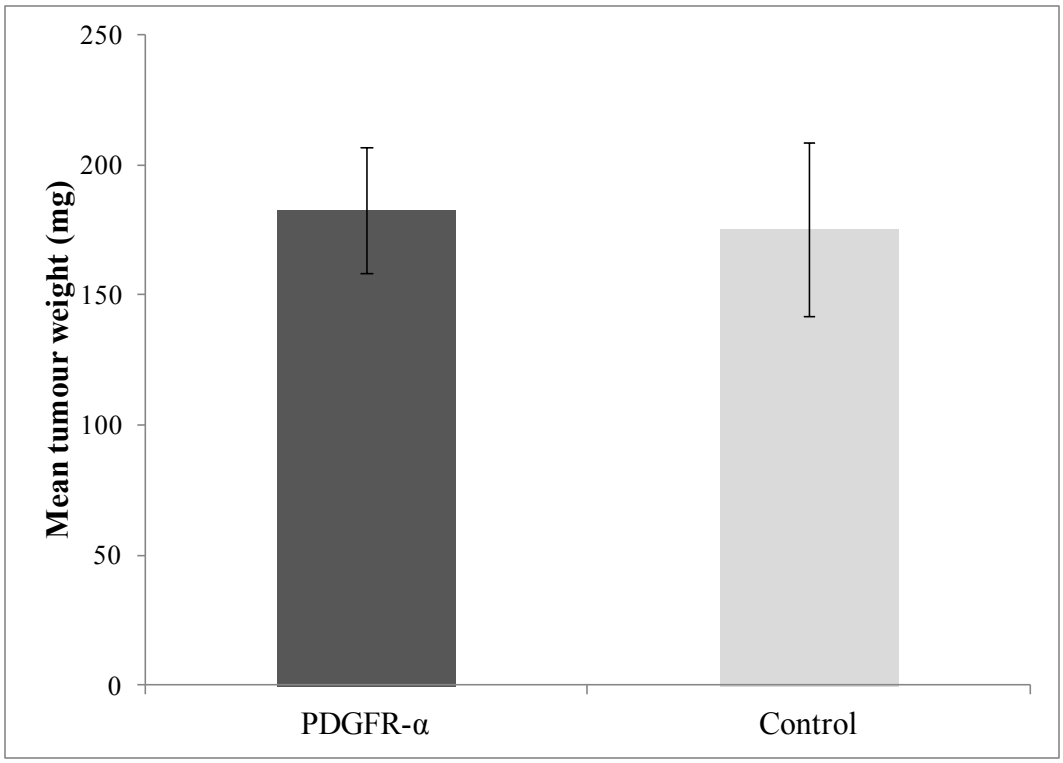


Figure 2.19. Mean tumour weight comparing PDGFR-α expressing BON-1 cells to empty vector control. There were no significant differences in tumour weight between the two groups.

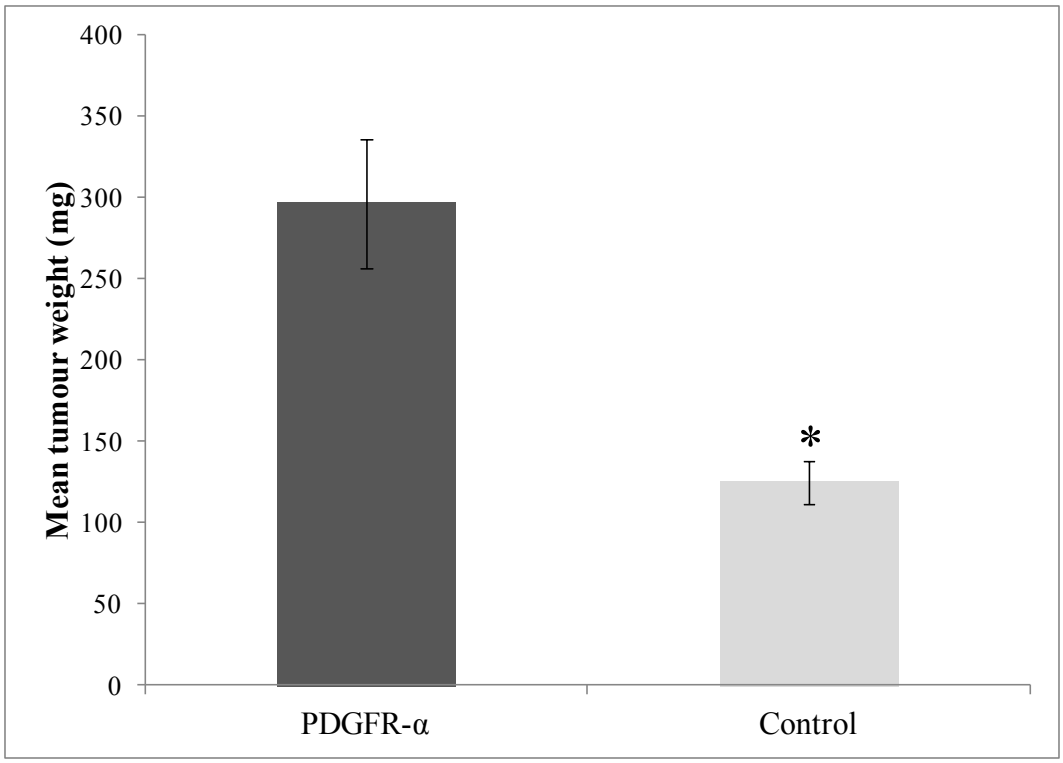


Figure 2.20. Mean tumour weight comparing PDGFR-α expressing QGP-1 cells to empty vector control. PDGFR-α expressing QGP-1 developed significantly larger tumour. * = $p < 0.05$

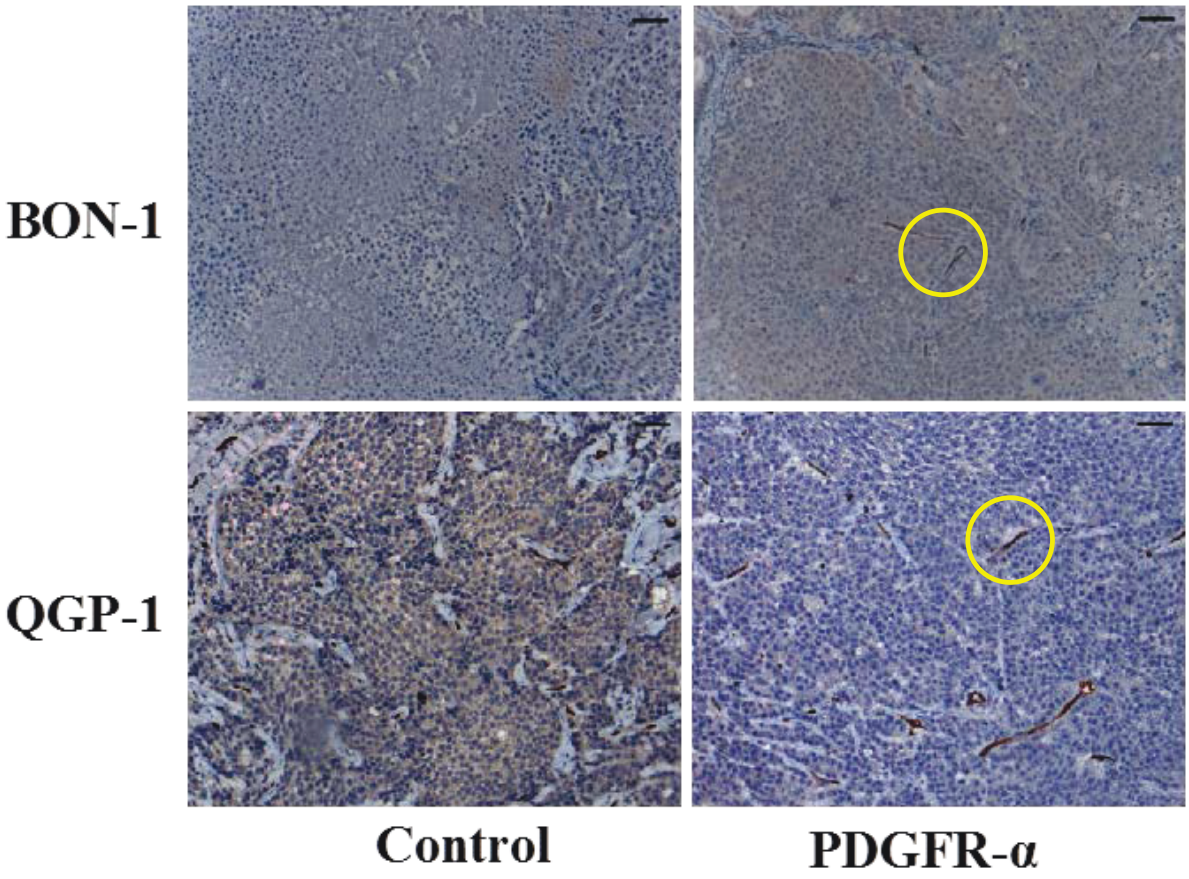
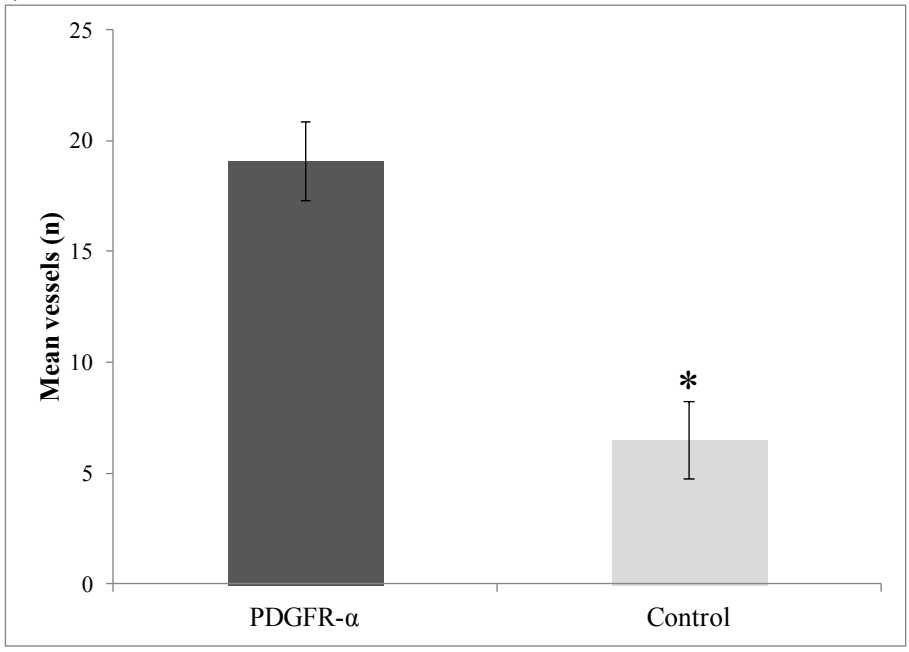


Figure 2.21. Representative immunohistochemical stains of anti-CD-31 at 20X magnification. Staining demonstrates a much higher vascular density in PDGFR-α expressing BON-1 and QGP-1 xenografts than the control. (50μm scale bar)

a)



b)

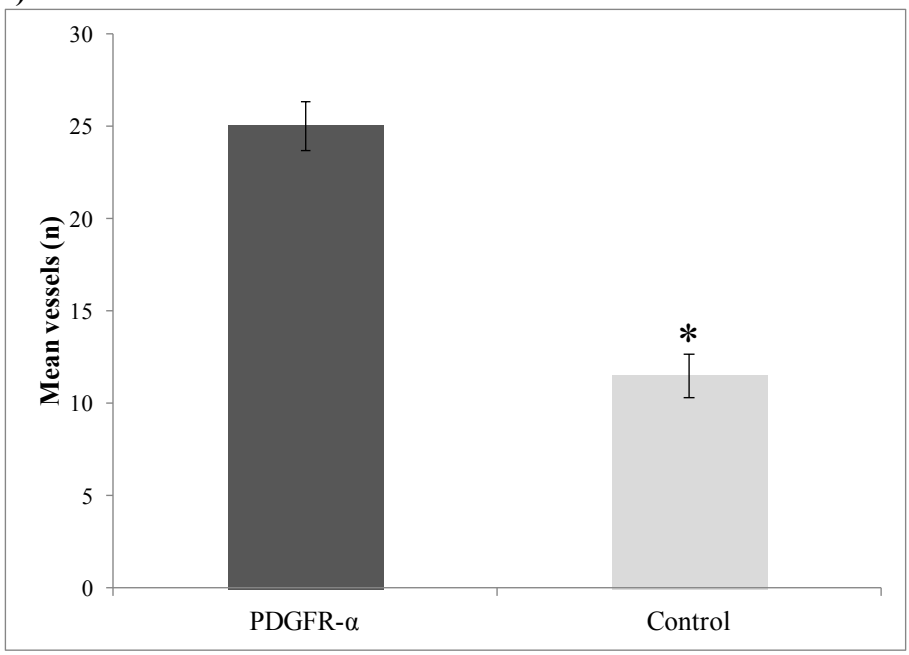


Figure 2.22. Microvascular density in mouse xenograft. Average number of blood vessels in **a)** BON-1 and **b)** QGP-1 xenografts comparing PDGFR-α expressing cells to empty vector control. Both PDGFR-α expressing BON-1 and QGP-1 cells developed significantly more blood vessels than the control. * = $p < 0.05$

2.3.6. PDGFR-α activation and increased migratory potential is mediated by both the MAPK/ERK and PI3K/Akt pathways

Current models for receptor tyrosine kinase signaling involve both the MAPK/ERK and PI3K/Akt pathways. The role of the MAPK/ERK and PI3K/Akt pathways in NET was examined using both BON-1 and QGP-1 cell lines. A link was demonstrated between PDGFR-α activation and downstream up-regulation of both the MAPK/ERK and PI3K/Akt pathways. PDGF-BB (50ng/ml) stimulation leads to increased levels of phospho- PDGFR-α, phospho-Akt, and phospho-ERK (Fig.2.23.). The addition of crenolanib, which abated changes in migratory potential in BON-1 cell line, completely blocked activation of PDGFR-α with corresponding significantly decreased activity in the PI3K/Akt pathway (Fig. 2.16.). Using pharmacologic blockade of either the PI3K/Akt or MAPK/ERK pathway in BON-1 cells, it was evident that both pathways are important in mediating the increased migratory potential. With either PI3K/Akt blockade (LY294002) or MAPK/ERK blockade (U0126), it was clear that the enhanced migratory potential in PDGFR-α expressing BON-1 was disrupted (Fig.2.24. and 2.25.).

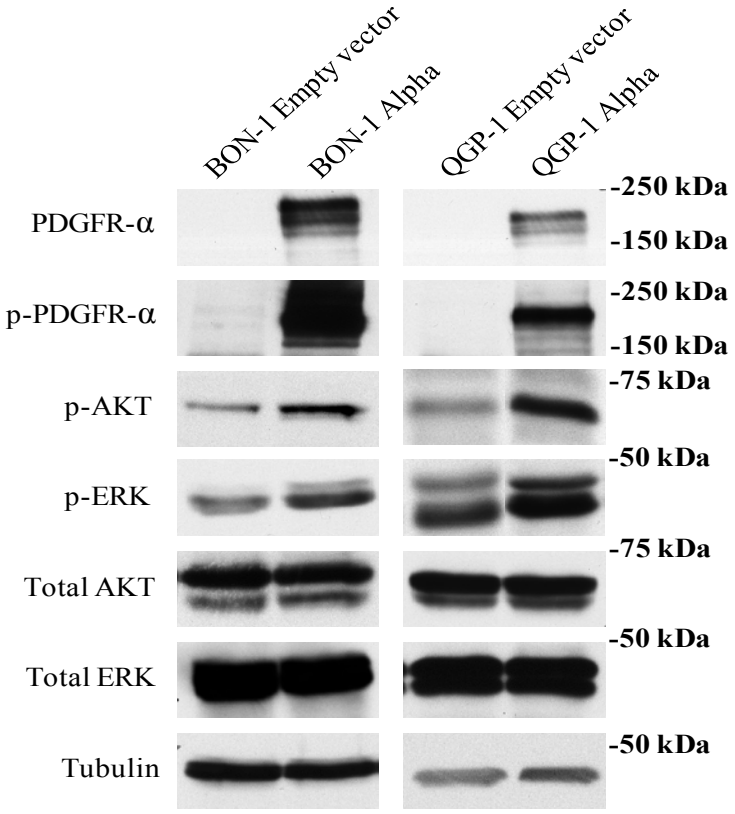


Figure 2.23. Activation of MAPK/ERK and PI3K/Akt pathways. Western blots for BON-1 and QGP-1 demonstrating up-regulation of downstream MAPK/ERK and PI3K/Akt pathways upon activation of PDGFR-α. PDGF-BB (50ng/ml) was added to the culture media.

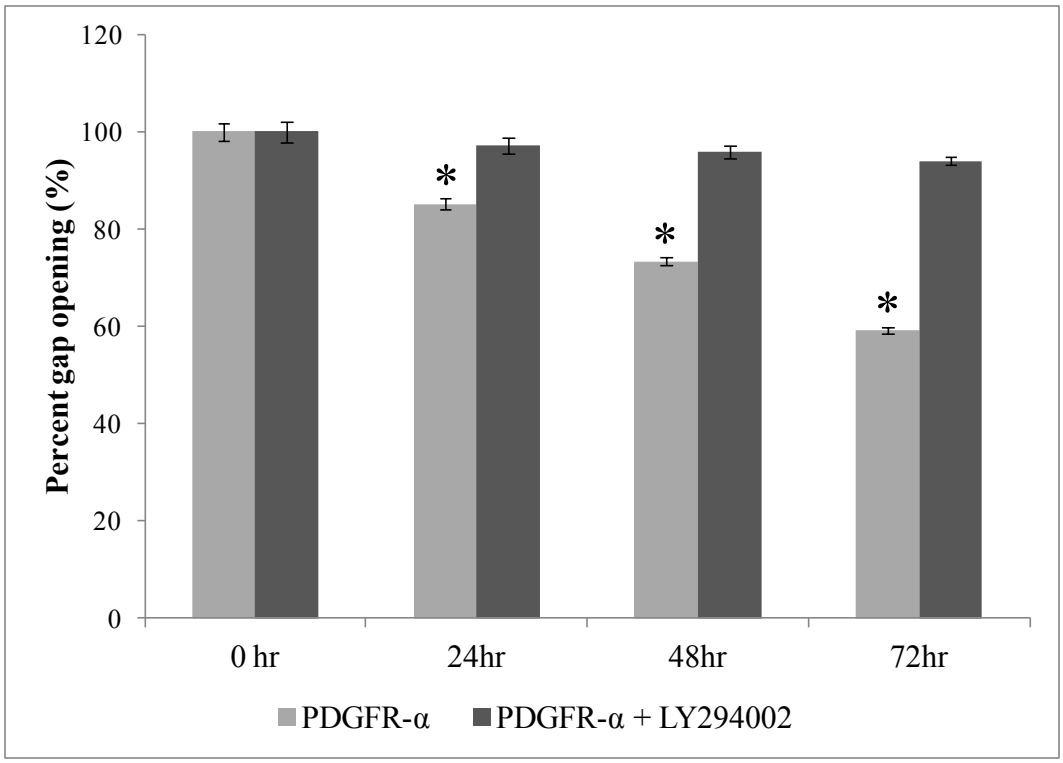


Figure 2.24. Inhibition of enhanced BON-1 migration by PI3K/AKT inhibitor (LY294002). The increased migratory potential observed in PDGFR-α expressing BON-1 cells was reversed by LY294002 treatment. PDGF-BB (50ng/ml) was added to the culture media. * = $p < 0.05$

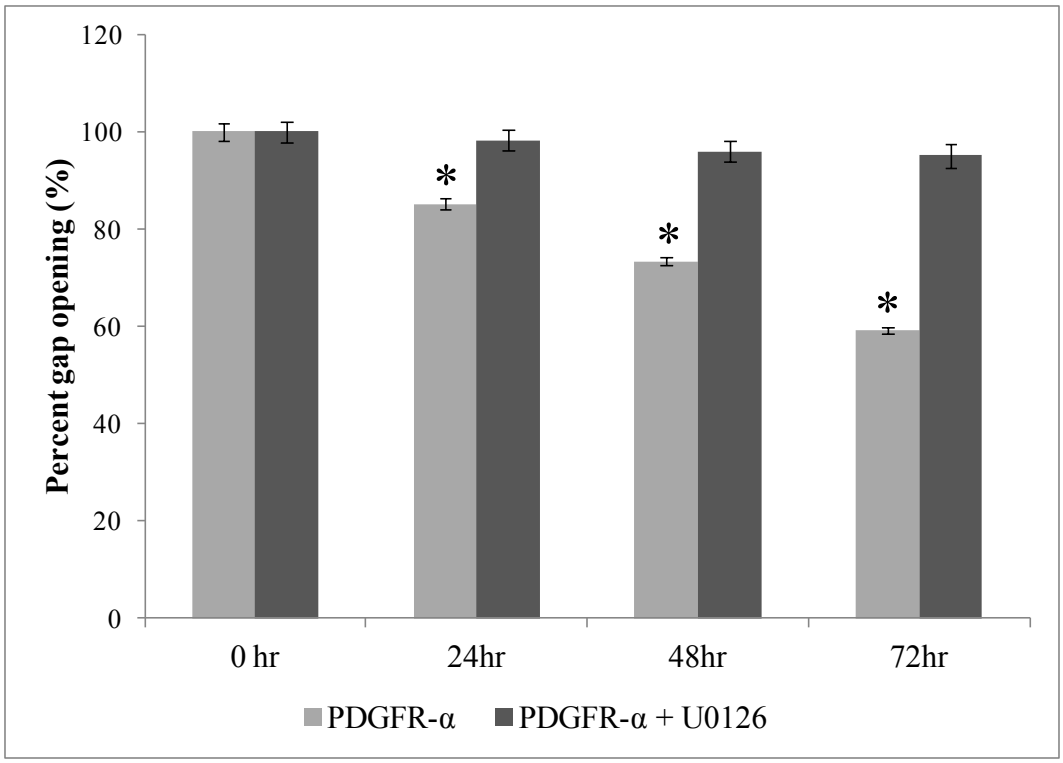


Figure 2.25. Inhibition of enhanced BON-1 migration by MEK inhibitor (U0126). The increased migratory potential observed in PDGFR-α expressing BON-1 cells was reversed by U0126 treatment. PDGF-BB (50ng/ml) was added to the culture media. * = $p < 0.05$

2.3.7. PDGFR-α activation is associated with epithelial-mesenchymal transition (EMT) in BON-1

An EMT is a cellular process during which epithelial cells lose their apical/ basal polarity and cell-cell adhesion complexes, and undergo multiple structural changes to assume a mesenchymal phenotype characterized by a fibroblast-like morphology and enhanced migratory capacity. During metastasis, tumour cells undergo EMT to invade surrounding vasculature and to migrate to distant target organs. During EMT, the expressions of various cellular structural proteins and genes are altered. For example, snail and vimentin are known to be increased in cells undergoing EMT. PDGFR-α expressing BON-1 had increased expression of snail, vimentin, and ZEB-1 comparing to the control. In addition, the expression of E-cadherin was also reduced upon PDGFR-α activation (Fig. 2.26.).

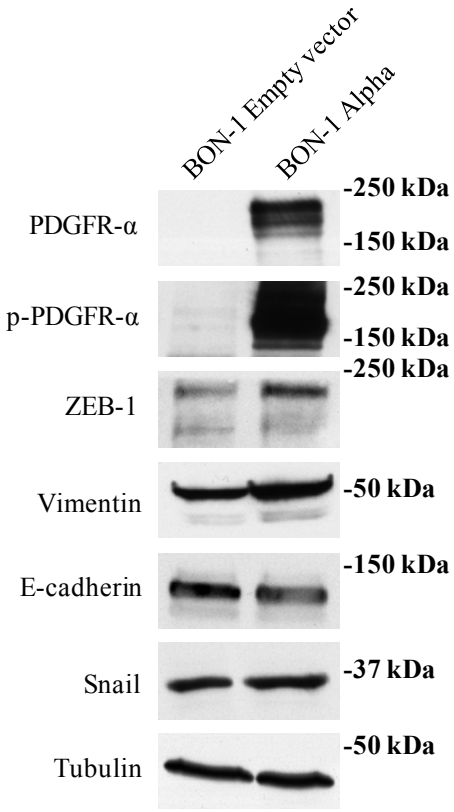


Figure 2.26. PDGFR-α activation and EMT. Western blots for BON-1 looking specifically at various key components of EMT. The expressions of snail, vimentin, and zeb-1 were increased upon PDGFR-α activation, whereas the expression of E-cadherin was reduced. PDGF-BB (50ng/ml) was added to the culture media.

2.3.8. PDGFR-α activation is associated with up-regulation of multiple genes involved in the malignant progression of NET

A customized 384-well qRT-PCR profiler was used to quantify the relative mRNA expression in BON-1 cell line, BON-1 xenograft, and in clinical NET specimens looking at the downstream effect of PDGFR-α activation. For BON-1 cell cultures and xenografts, the comparison was made between the PDGFR-α expressing BON-1 and the empty vector control. For the clinical NET specimens, the comparison was made between metastatic liver lesions (n = 5) and primary tumours (n = 5). Of the 188 gene targets that have been surveyed, a total of 7 gene candidates, namely the FGF-1, IGFBP-3, OPN, XIAP, FAS ligand, Autotaxin, and LPAR-4, appears to demonstrate consistent changes across different samples ranging from *in vitro* cell cultures to *in vivo* xengrafts and ultimately in clinical NET specimens (Fig. 2.27.).

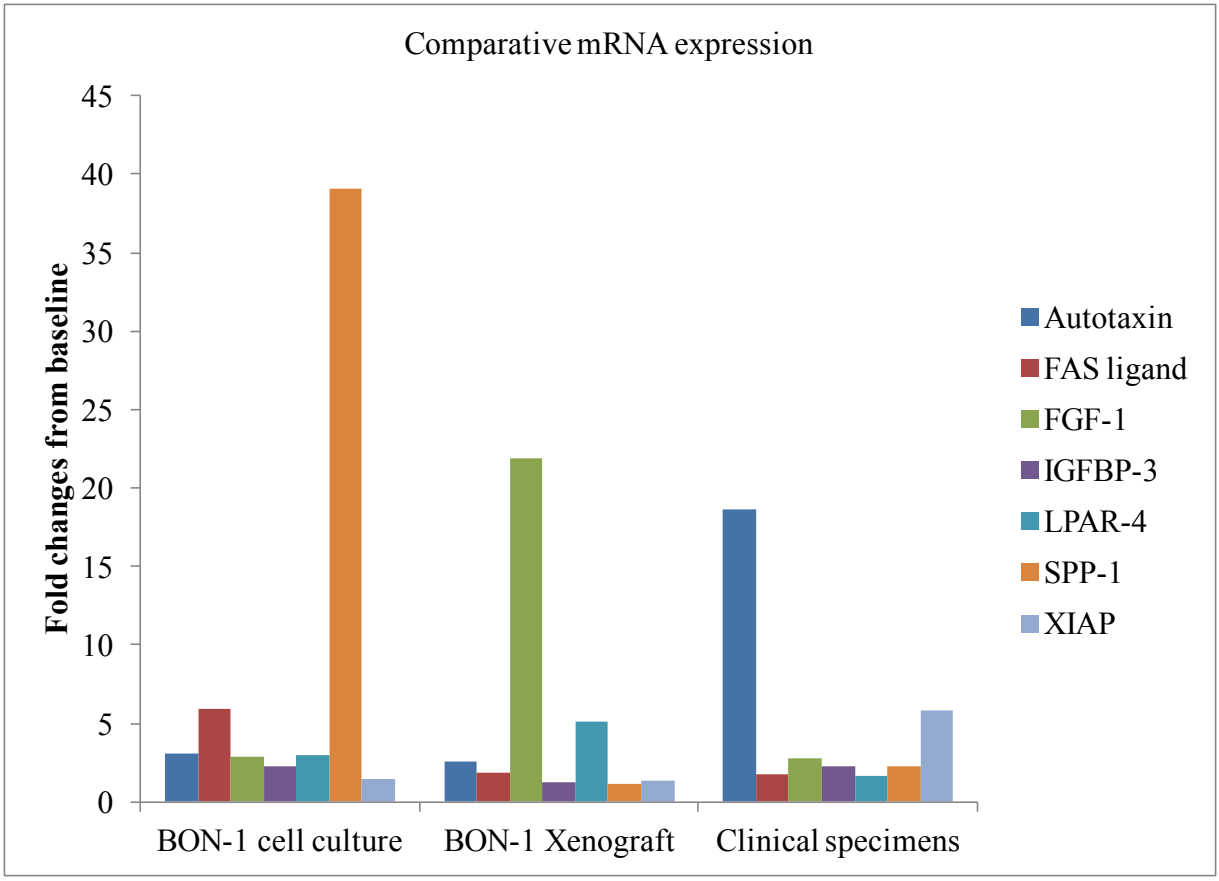


Figure 2.27. PDGFR-α activation and downstream gene upregulation. Comparative analysis of mRNA expression among various samples using qRT-PCR. A 2-fold increase in mRNA expression is used as threshold for the analysis.

2.4. Discussion

In the present study, we sought to investigate the role of PDGFR-α in the disease progression of NETs as well as to generate an *in vitro* model that closely resembles clinical specimens. The main findings are as follows: i) PDGFR-α increases migratory potential in BON-1 *in vitro*; ii) Selective disruption of PDGFR-α signaling cascade abates the migratory phenotype; iii) PDGFR-α activation and enhanced migratory phenotype is mediated by both the MAPK/ERK and PI3K/Akt pathways; iv) activation of PDGFR-α boosts tumour angiogenesis and growth *in vivo*; vi) PDGFR-α expression in both BON-1 and QGP-1 cell lines enhances their application as *in vitro* models for the study of NETs.

NETs are highly vascular and angiogenesis plays an important role in their development and progression. Expression of vascular endothelial growth factor (VEGF) and its receptors have previously been demonstrated in both gastro-intestinal and pulmonary NETs [19]. Treatment with either the VEGF monoclonal antibody or interferon- α (suppression of VEGF gene transcription) in mouse model resulted in reduction of metastatic burden and micro-vascular density, respectively [103, 110]. However, there are limited studies on other pro-angiogenic factors such as the PDGF/PDGFR which are also expressed by NETs. Our results demonstrated that PDGFR-α was expressed in clinical NET specimens, but not in the BON-1 and QGP-1 cell lines. The finding of PDGFR-α expression in clinical specimens has led us to hypothesize that the activation of PDGFR-α promotes tumour metastasis in NET. Given the scarcity of other available neuroendocrine cell lines and the importance of PDGFR-α expression in mediating tumour metastasis in other solid tumours, stable clones with inducible PDGFR-α expression were established to further investigate its role in mediating tumour metastasis in NETs.

In this present study, we demonstrated that the activation of PDGFR-α in BON-1 increases its migratory potential *in vitro*, but revealed no statistically significant difference in tumourigenicity in *vivo* despite demonstrating higher microvascular density. The reason for such discrepancy can be elusive. It could be that tumour weight by itself may not be a full representation of its metastatic potential. More importantly, since the mouse xenograft model is not orthotopic, it is likely that the PDGFR-α expressing BON-1 is not able to exert its full malignant potential when taken out of its natural tissue microenvironment. PDGFR-α expressing QGP-1 cells, on the other hand, grew significantly larger tumours in mouse xenografts, but failed to demonstrate such aggressive phenotype *in vitro*. It is well established that essential cellular functions/interactions are missed by conventional 2-dimenional (2D) cell cultures and therefore limit their potential to predict the cellular responses in animal models [111]. This lack of *in vitro* differences (despite showing trends favoring PDGFR-α expressing QGP-1) in metastatic behaviors could partly be explained by the loss of supporting tumour stroma in 2D cell culture.

To further strengthen our study, we also used siRNA to disrupt expression of PDGFR-α in primary cell cultures (n=3) generated from clinical specimens exhibiting PDGFR-α and examined the subsequent effect on their migratory potential. A consistent reduction in migratory potential was observed in all treatment groups signifying the importance of PDGFR-α expression in mediating tumour metastasis. In addition, this present study also revealed that the increased migratory potential mediated by PDGFR-α signaling requires the activity of both the MAPK/ERK and PI3K/Akt downstream pathways. Blockade of PDGFR-α signaling either at the receptor-level using crenolanib or at downstream pathway using Ly294002 and U0126 can abate the migratory phenotype.

Metastatic diseases account for more than 90% cancer-related mortality [112]. It is of utmost clinical relevance to prevent metastatic formation as well as to target existing disease. Tumour metastasis occurs in a step-wise fashion starting with local invasion, intravasation, systemic transport, and finally extravasation followed by colonization at distant site. Many recent studies have provided evidence of EMT, a process of switching polarized epithelial cells to a migratory mesenchymal type of cells, at operation during each step of tumour metastasis [112, 113]. In this present study, it was demonstrated for the first time that activation of PDGFR-α in BON-1 is associated with EMT further supporting the role of PDGFR-α in mediating a more aggressive phenotype.

To further characterize the downstream effect of PDGFR-α activation in promoting metastatic phenotype at the gene level, we conducted a preliminary study using a customized 384-well qRT-PCR profiler to quantify the relative mRNA expression in various BON-1 constructs (PDGFR-α *v.s.* Empty vector) as well as in clinical specimens (Metastatic *v.s.* Primary lesion). Multiple genes were consistently up-regulated across all sample ranges. Among those up-regulated genes, many are known to be involved in the malignant progression of other solid tumours. More importantly, there are existing small molecule inhibitors for these targets already. Such information would be beneficial to oncologists in selecting the appropriate combinational therapy to treat patients.

Better understandings of the underlying molecular biology in NET have recently led to the development of targeted therapy for this group of diseases. The rationale for selecting tyrosine kinase inhibitors (TKIs) in treating NETs, including atipromid, gefitnib, imatinib, sorafenib, and

sunitinib, has essentially been empirical [114]. Many of these TKIs are multi-kinase inhibitors that target the different PDGF, VEGF, and EGF receptors and it is often difficult to find out which receptor subgroup is most effectively targeted. Differences in PDGFR or VEGFR targeting by TKIs may account for the varied responses to TKI therapy in NETs. Based on our current results, the relative success of sunitinib and everolimus may be due in part to its targeting of PDGFR and its downstream signal mediator mTOR, respectively.

In summary, PDGFR-α appears to confer increased migratory potential in neuroendocrine cell lines. Downstream signaling is mediated through both the MAPK/ERK and PI3K/Akt pathways and disruption of either pathway can mitigate the effects of PDGFR-activation on cell migration. Based on our results, tyrosine kinase inhibitors that target PDGFR, specifically the α-subunit, may be better suited to treating metastatic NETs.

Chapter 3: Discussion and Future Directions

In this present study, it was found that the expression of PDGFR-α in BON-1 enhanced its migratory potential in vitro and this migratory phenotype could be reversed by selective inhibition of PDGFR-α signaling. This experimental finding was further confirmed in primary cell cultures using PDGFR-α specific siRNA knockdown suggesting a role of PDGFR-α in tumourigenesis. Despite the lack of *in vivo* growth enhancement in PDGFR-α expressing BON-1, the expression of PDGFR-α did promote angiogenesis and growth in BON-1 and QGP-1 *in vivo*, respectively. Similarly, the expression of PDGFR-α also enhanced the colony-formation capability of both BON-1 and QGP-1. Collectively, all these experimental findings are supportive of PDGFR-α being a potential therapeutic target. Now that we have characterized the phenotypes for both cell lines, we can evaluate the effect of PDGFR-α inhibition both *in vitro* and *in vivo* such that a model for drug screening can be developed ultimately leading towards a clinical trial.

3.1. PDGFR-α as a diagnostic and therapeutic target

Molecular imaging emerged in the early twenty-first century as a field integrating molecular biology to *in vivo* imaging and is one of the most rapidly growing area of science. Molecular imaging differs from traditional imaging in that probes known as biomarkers are used to image particular targets or pathways. Conventional imaging techniques rely primarily on detecting differences in qualities such as density or water content whereas molecular imaging highlights the chemical interactions between biomarkers and their surroundings. This ability to image fine molecular changes opens up an incredible number of exciting possibilities for medical application, including early detection and treatment of disease.

Immuno-positron emission tomography (PET) is a new molecular imaging tool that combines PET with monoclonal antibodies (mAbs). The application of mAb offers the unique ability to selectively target specific tumour antigens. Such an arrangement not only utilizes the high sensitivity and resolution of a PET, but also the specificity of a mAb. Immuno-PET is like performing a real time *in vivo* immunohistochemical staining [115]. In the setting of personalized medicine, immune-PET enables the confirmation of tumour targeting as well as the quantification of mAb accumulation. In doing so, future patients can be screened for presence or absence of certain tumour antigen in a non-invasive fashion reducing the risk of false negative receptor expression from non-representative biopsies. Given that the majority of NETs expresses PDGFR-α, the application immuno-PET using mAb against PDGFR-α can serve as an alternative imaging modality for patients with non-MIBG, non-octreotide avid lesions. In addition, immune-PET can also be used for therapeutic purposes by radiolabeling mAbs with high energy α/β-emitting radio-isotopes and thereby enabling them to selectively deliver radiation to tumour cells. For future studies, it would be important to determine the level of PDGFR-α expression in non-cancerous tissues. In doing so, tumour-to-background ratios can be measured to determine the feasibility of radioimmunotherapy.

3.2. Clinical trial to evaluate the efficacy of PDGFR-α inhibition in NETs

Based on our current results, the relative success of sunitinib and everolimus may be due in part to its targeting of PDGFR and its downstream signal mediator mTOR, respectively. We would propose that selective inhibition of PDGFR-α, which is widely expressed in NETs, may be a better therapeutic target. To this end, we would conduct a prospective, multicenter, randomized,

double-blind, placebo-controlled, phase III study comparing the efficacy and safety of PDGFR-α inhibition (monoclonal antibody or small-molecule inhibitor) plus octreotide LAR to placebo plus octreotide LAR. The inclusion criteria of this study would be: patients that are 18 years of age or older, with low-to-intermediate grade unresectable/metastatic NETs, radiologic evidence of disease progression within the past 12 months, and history of clinical symptoms such as diarrhea and flushing. Patients would then be assigned to receive PDGFR-α inhibitor or matching placebo in conjunction with 30mg octreotide LAR every 28 days. Treatment would continue until disease progression, development of unacceptable toxicity, or withdrawal of consent. Patients randomly assigned to placebo plus octreotide LAR can cross-over to treatment arm at the time of investigator determination of disease progression. The primary endpoint of this study would be progression-free survival, defined as the time from randomization to the first documentation of disease progression or death from any cause. The secondary endpoints would be objective response rate, biomarker assessment, overall survival and safety. Serum chromogranin A and urinary 5-HIAA would be collected at baseline. Tumours would be measured at baseline and every 3 months thereafter. Safety evaluation would include routine physical examination monthly, regular monitoring of hematologic and clinical biochemistry levels, and monitoring of adverse events.

3.3. PDGFR-α induced EMT and tumour metastasis

To investigate the underlying molecular biology involved in the enhanced migratory phenotype in PDGFR-α expressing BON-1, we sought to study the relationship between PDGFR-α activation and epithelial-mesenchymal transition (EMT). EMT is a cellular process during which

epithelial cells lose their apical/ basal polarity and cell-cell adhesion complexes, and undergo multiple structural changes to assume a mesenchymal phenotype characterized by a fibroblast-like morphology and enhanced migratory capacity [113]. EMT can be induced by a number of extra-cellular signals and many of these EMT inducing signals are cell-type/ tissue specific. In addition, many transcription factors that regulate the gene expression required for EMT program have also been identified. To date, three core groups of transcriptional regulators have been consistently shown to be critical during EMT. The first group is the Snail family of zinc-finger transcription factors, including Snail-1 and Snail-2 [116]. The second group is the zinc-finger E-box-binding homeobox family proteins, Zeb 1 and Zeb 2 [117]. The last group consists of Twist 1, Twist 2, and E12/ E47 from the basic helix-loop-helix family of transcription factors which are also capable of inducing EMT [118, 119].

During EMT, the expressions of various cellular structural proteins are altered. EMT involves the loss of epithelial markers such as the claudins, occludins, and E-cadherin [120, 121]. In addition, a switch of intermediate filament from cytokeratin to vimentin is another hallmark of EMT. Increased expression of vimentin has been a consistent marker during various EMT. Other key EMT effectors involve the expression of proteins that enhance cell migration and invasion including N-cadherin, fibronectin, and matrix metalloproteinases (MMPs). In this present study, it was demonstrated for the first time that activation of PDGFR-α in BON-1 is associated with EMT resulting in a more aggressive phenotype. Autocrine PDGF/PDGFR signaling was first observed to be necessary in the maintenance of EMT by Jechlinger et al [122]. In their study, PDGFR signaling was found to be crucial for experimental metastasis in mice. More recently, PDGFR-α signaling has also been implicated in the formation of invadopodia which are actin-

based protrusion that can recruit various proteases to degrade extracellular matrix and thereby facilitate tumour invasion. Of interest, it was also noted that the morphology of PDGFR-α expressing BON-1 differed considerably from the empty-vector control presenting with more cell surface protrusions. Tumour metastasis is the major cause of cancer morbidity, but strategies for direct interference with invasion processes are lacking. The results from our study suggest that PDGFR-α might serve as a rational target for therapeutic intervention. For future studies, it would be important to show that selective inhibition of PDGFR-α with either small molecule inhibitor or siRNA can indeed reverse the process of EMT. Moreover, given the morphological changes seen in PDGFR-α expressing BON-1, further studies are warranted to investigate changes in the underlying cytoskeletal structure as well as cellular adhesion complex.

3.4. PDGFR-α induced downstream gene up-regulation

Thus far, we have demonstrated that the activation of PDGFR-α leads to an aggressive phenotype both *in vitro* and *in vivo*. However, not much is known about the cellular mechanisms or mediators involved in such transformation. To further delineate the downstream effect of PDGFR-α activation, a customized 384-well qRT-PCR array profiler was used to compare the relative mRNA expression in various BON-1 constructs. Multiple genes known to be involved in malignant progression of other solid tumours were found to be up-regulated. Of the 188 gene targets that have been surveyed, a total of 7 gene candidates, namely the FGF-1, IGFBP-3, OPN, XIAP, FAS ligand, Autotaxin, and LPAR-4, appears to be consistently up-regulated. It is of interest that both autotaxin (ATX) and LPA receptor 4 were up-regulated suggesting a possible ATX/LPA autocrine signalling loop. Autotaxin is a secreted enzyme that produces most of the extracellular LPA which mediates many physiological and pathological processes by signaling

through at least six G-protein coupled receptors (LPAR-1 to 6) [123]. LPA is known to increase vascular endothelial growth factor (VEGF) production, which stimulates angiogenesis, a process necessary for tumour progression [124]. LPA also decreases the expression of the tumour suppressor p53, thus enhancing tumour cell survival [125]. Moreover, mouse model over-expressing ATX and LPA in mammary epithelium develop spontaneous metastatic mammary tumours [126]. It is likely that aberrant ATX/LPA signaling has a significant contribution to the PDGFR-α mediated increase in metastatic behaviour. Such information would be beneficial to oncologists in selecting the appropriate duel-target (ATX and PDGFR-α inhibitor) therapy to treat patients. However, this is a preliminary study with relatively small sample size (n = 5), a confirmatory study has to be repeated on a larger cohort of clinical specimens and western blot analysis has to be done to demonstrate the parallel up-regulation at the protein level.

Cancer therapy, in the setting of metastatic disease, should selectively induce tumour cell death leading to disease regression and ultimately patient cure. However, tumour cells often develop multiple protective mechanisms to resist apoptosis leading to disease relapse and treatment resistance despite therapy. Recent evidences have indicated an important role of XIAP and FAS ligand in resisting cell death and evading immune attack, respectively. XIAP, the X-linked inhibitor of apoptosis, acts as an endogenous suppressor of cellular apoptosis and it exerts its anti-apoptotic activity by limiting the activity of several crucial death-inducing caspases including caspase-3, -7, and -9 [127]. In addition, XIAP also blocks both the extrinsic and intrinsic death pathways and thereby preventing cell death. Over-expression of XIAP is associated with disease progression and treatment resistance in various malignancies [127, 128]. FAS ligand, on the other hand, is a type II transmembrane protein belonging to the tumour

necrosis factor (TNF) family. Activated cytotoxic T cells express FAS receptor and are susceptible to FAS-mediated apoptosis [129]. The discovery that FAS ligand is expressed by a variety of tumour cells raised the possibility that FAS ligand may participate in tumour immune evasion. Over-expression of FAS ligand by tumour cells may enable them to kill infiltrating lymphocytes and thereby protecting tumour cells from immune responses. In fact, numerous studies have shown that FAS ligand expressing tumour cells is capable of killing FAS-bearing cells *in vitro* [130, 131] and loss of tumour-infiltrating lymphocytes *in vivo* [132, 133]. In this pilot study, it was demonstrated that the expression levels of XIAP and FAS ligand in PDGFR-α bearing cells and metastatic lesions are significantly higher suggesting that these tumours cells are more resistant to apoptosis and are more capable of evading immune surveillance. For future studies, it would be interesting to investigate the downstream signaling pathways leading to the up-regulation of XIAP and FAS ligand. In addition, it would be important to study whether or not treatment sensitivity in chemo-resistant tumours can be restored by inhibiting XIAP.

References

[1] R.A. DeLellis, The neuroendocrine system and its tumors: an overview, Am J Clin Pathol 115 Suppl (2001) S5-16.

[2] K. Oberg, Neuroendocrine gastrointestinal tumors--a condensed overview of diagnosis and treatment, Ann Oncol 10 Suppl 2 (1999) S3-8.

[3] K. Langley, The neuroendocrine concept today, Ann N Y Acad Sci 733 (1994) 1-17.

[4] A.G. Pearse, T. Takor, Embryology of the diffuse neuroendocrine system and its relationship to the common peptides, Fed Proc 38 (1979) 2288-2294.

[5] N.M. Le Douarin, M.A. Teillet, The migration of neural crest cells to the wall of the digestive tract in avian embryo, J Embryol Exp Morphol 30 (1973) 31-48.

[6] J.C. Yao, M. Hassan, A. Phan, C. Dagohoy, C. Leary, J.E. Mares, E.K. Abdalla, J.B. Fleming, J.N. Vauthey, A. Rashid, D.B. Evans, One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States, J Clin Oncol 26 (2008) 3063-3072.

[7] G.A. Kaltsas, G.M. Besser, A.B. Grossman, The diagnosis and medical management of advanced neuroendocrine tumors, Endocr Rev 25 (2004) 458-511.

[8] M. Mignon, Natural history of neuroendocrine enteropancreatic tumors, Digestion 62 Suppl 1 (2000) 51-58.

[9] M.H. Kulke, J. Bendell, L. Kvols, J. Picus, R. Pommier, J. Yao, Evolving diagnostic and treatment strategies for pancreatic neuroendocrine tumors, J Hematol Oncol 4 (2011) 29.

[10] I.M. Modlin, K. Oberg, D.C. Chung, R.T. Jensen, W.W. de Herder, R.V. Thakker, M. Caplin, G. Delle Fave, G.A. Kaltsas, E.P. Krenning, S.F. Moss, O. Nilsson, G. Rindi, R. Salazar, P. Ruszniewski, A. Sundin, Gastroenteropancreatic neuroendocrine tumours, Lancet Oncol 9 (2008) 61-72.

[11] R. G, A. R, B. FT, Nomenclature and classification of neuroendocrine neoplasms of the digestive system, in: B. FT, C. F, H. RH, T. ND (Eds.) WHO classification of tumors of the digestive system., IARC, Lyon, 2010.

[12] M. Anlauf, N. Garbrecht, J. Bauersfeld, A. Schmitt, T. Henopp, P. Komminoth, P.U. Heitz, A. Perren, G. Kloppel, Hereditary neuroendocrine tumors of the gastroenteropancreatic system, Virchows Archiv : an international journal of pathology 451 Suppl 1 (2007) S29-38.

[13] S.J. Marx, W.F. Simonds, Hereditary hormone excess: genes, molecular pathways, and syndromes, Endocr Rev 26 (2005) 615-661.

[14] Y. Dayal, K.A. Tallberg, G. Nunnemacher, R.A. DeLellis, H.J. Wolfe, Duodenal carcinoids in patients with and without neurofibromatosis. A comparative study, The American journal of surgical pathology 10 (1986) 348-357.

[15] E.R. Woodward, E.R. Maher, Von Hippel-Lindau disease and endocrine tumour susceptibility, Endocr Relat Cancer 13 (2006) 415-425.

[16] E.S. Roach, M.R. Gomez, H. Northrup, Tuberous sclerosis complex consensus conference: revised clinical diagnostic criteria, J. Child Neurol. 13 (1998) 624-628.

[17] E.S. Roach, F.J. DiMario, R.S. Kandt, H. Northrup, Tuberous Sclerosis Consensus Conference: recommendations for diagnostic evaluation. National Tuberous Sclerosis Association, J. Child Neurol. 14 (1999) 401-407.

[18] P.D. Leotlela, A. Jauch, H. Holtgreve-Grez, R.V. Thakker, Genetics of neuroendocrine and carcinoid tumours, Endocr Relat Cancer 10 (2003) 437-450.

[19] J.C. Yao, Neuroendocrine tumors. Molecular targeted therapy for carcinoid and islet-cell carcinoma, Best practice & research. Clinical endocrinology & metabolism 21 (2007) 163-172.

[20] H. Kim do, Y. Nagano, I.S. Choi, J.A. White, J.C. Yao, A. Rashid, Allelic alterations in well-differentiated neuroendocrine tumors (carcinoid tumors) identified by genome-wide single nucleotide polymorphism analysis and comparison with pancreatic endocrine tumors, Genes. Chromosomes Cancer 47 (2008) 84-92.

[21] E. Brambilla, A. Negoescu, S. Gazzeri, S. Lantuejoul, D. Moro, C. Brambilla, J.L. Coll, Apoptosis-related factors p53, Bcl2, and Bax in neuroendocrine lung tumors, The American journal of pathology 149 (1996) 1941-1952.

[22] T.K. Zirbes, J. Lorenzen, S.E. Baldus, S.P. Moenig, U. Wolters, A. Ottlik, J. Thiele, A.H. Holscher, H.P. Dienes, Apoptosis and expression of bcl-2 protein are inverse factors influencing tumour cell turnover in primary carcinoid tumours of the lung, Histopathology 33 (1998) 123-128.

[23] B. Lawrence, B.I. Gustafsson, A. Chan, B. Svejda, M. Kidd, I.M. Modlin, The epidemiology of gastroenteropancreatic neuroendocrine tumors, Endocrinol. Metab. Clin. North Am. 40 (2011) 1-18, vii.

[24] A. Faggiano, P. Ferolla, F. Grimaldi, D. Campana, M. Manzoni, M.V. Davi, A. Bianchi, R. Valcavi, E. Papini, D. Giuffrida, D. Ferone, G. Fanciulli, G. Arnaldi, G.M. Franchi, G. Francia, G. Fasola, L. Crino, A. Pontecorvi, P. Tomassetti, A. Colao, Natural history of gastro-entero-pancreatic and thoracic neuroendocrine tumors. Data from a large prospective and retrospective Italian epidemiological study: the NET management study, J. Endocrinol. Invest. 35 (2012) 817-823.

[25] G. Kaltsas, Androulakis, II, W.W. de Herder, A.B. Grossman, Paraneoplastic syndromes secondary to neuroendocrine tumours, Endocr Relat Cancer 17 (2010) R173-193.

[26] M. Druce, A. Rockall, A.B. Grossman, Fibrosis and carcinoid syndrome: from causation to future therapy, Nature reviews. Endocrinology 5 (2009) 276-283.

[27] J.L. Cunningham, E.T. Janson, S. Agarwal, L. Grimelius, M. Stridsberg, Tachykinins in endocrine tumors and the carcinoid syndrome, Eur. J. Endocrinol. 159 (2008) 275-282.

[28] A.J. van der Lely, W.W. de Herder, Carcinoid syndrome: diagnosis and medical management, Arq. Bras. Endocrinol. Metabol. 49 (2005) 850-860.

[29] K. Mancuso, A.D. Kaye, J.P. Boudreaux, C.J. Fox, P. Lang, P.L. Kalarickal, S. Gomez, P.J. Primeaux, Carcinoid syndrome and perioperative anesthetic considerations, J. Clin. Anesth. 23 (2011) 329-341.

[30] E. Batcher, P. Madaj, A.G. Gianoukakis, Pancreatic neuroendocrine tumors, Endocr. Res. 36 (2011) 35-43.

[31] D. Vaidakis, J. Karoubalis, T. Pappa, G. Piaditis, G.N. Zografos, Pancreatic insulinoma: current issues and trends, Hepatobiliary & pancreatic diseases international : HBPD INT 9 (2010) 234-241.

[32] F. Gibril, M. Schumann, A. Pace, R.T. Jensen, Multiple endocrine neoplasia type 1 and Zollinger-Ellison syndrome: a prospective study of 107 cases and comparison with 1009 cases from the literature, Medicine (Baltimore). 83 (2004) 43-83.

[33] J.C. Mansour, H. Chen, Pancreatic endocrine tumors, The Journal of surgical research 120 (2004) 139-161.

[34] G. Xiang, X. Liu, C. Tan, H. Zhang, G. Mai, Z. Zheng, Diagnosis and treatment of VIPoma: a case report and literature review in China, Pancreas 41 (2012) 806-807.

[35] S.R. Bloom, J.M. Polak, A.G. Pearse, Vasoactive intestinal peptide and watery-diarrhoea syndrome, Lancet 2 (1973) 14-16.

[36] C.A. Proye, J.S. Lokey, Current concepts in functioning endocrine tumors of the pancreas, World J. Surg. 28 (2004) 1231-1238.

[37] M. Zhang, X. Xu, Y. Shen, Z.H. Hu, L.M. Wu, S.S. Zheng, Clinical experience in diagnosis and treatment of glucagonoma syndrome, Hepatobiliary & pancreatic diseases international : HBPD INT 3 (2004) 473-475.

[38] G.J. Harris, F. Tio, A.B. Cruz, Jr., Somatostatinoma: a case report and review of the literature, J. Surg. Oncol. 36 (1987) 8-16.

[39] B.I. Gustafsson, M. Kidd, I.M. Modlin, Neuroendocrine tumors of the diffuse neuroendocrine system, Curr. Opin. Oncol. 20 (2008) 1-12.

[40] A.I. Vinik, M.P. Silva, E.A. Woltering, V.L. Go, R. Warner, M. Caplin, Biochemical testing for neuroendocrine tumors, Pancreas 38 (2009) 876-889.

[41] I.M. Modlin, B.I. Gustafsson, S.F. Moss, M. Pavel, A.V. Tsolakis, M. Kidd, Chromogranin A--biological function and clinical utility in neuro endocrine tumor disease, Ann. Surg. Oncol. 17 (2010) 2427-2443.

[42] D. O'Toole, A. Grossman, D. Gross, G. Delle Fave, J. Barkmanova, J. O'Connor, U.F. Pape, U. Plockinger, ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Tumors: biochemical markers, Neuroendocrinology 90 (2009) 194-202.

[43] B. Eriksson, K. Oberg, M. Stridsberg, Tumor markers in neuroendocrine tumors, Digestion 62 Suppl 1 (2000) 33-38.

[44] M. Stridsberg, B. Eriksson, K. Oberg, E.T. Janson, A comparison between three commercial kits for chromogranin A measurements, The Journal of endocrinology 177 (2003) 337-341.

[45] M. Stridsberg, B. Eriksson, K. Oberg, E.T. Janson, A panel of 13 region-specific radioimmunoassays for measurements of human chromogranin B, Regul. Pept. 125 (2005) 193-199.

[46] S. Welin, M. Stridsberg, J. Cunningham, D. Granberg, B. Skogseid, K. Oberg, B. Eriksson, E.T. Janson, Elevated plasma chromogranin A is the first indication of recurrence in radically operated midgut carcinoid tumors, Neuroendocrinology 89 (2009) 302-307.

[47] M. Stridsberg, B. Eriksson, B. Fellstrom, G. Kristiansson, E. Tiensuu Janson, Measurements of chromogranin B can serve as a complement to chromogranin A, Regul. Pept. 139 (2007) 80-83.

[48] A. Abou-Saif, F. Gibril, J.V. Ojeaburu, S. Bashir, L.K. Entsuah, B. Asgharian, R.T. Jensen, Prospective study of the ability of serial measurements of serum chromogranin A and gastrin to detect changes in tumor burden in patients with gastrinomas, Cancer 98 (2003) 249-261.

[49] M. Stridsberg, K. Oberg, Q. Li, U. Engstrom, G. Lundqvist, Measurements of chromogranin A, chromogranin B (secretogranin I), chromogranin C (secretogranin II) and pancreastatin in plasma and urine from patients with carcinoid tumours and endocrine pancreatic tumours, The Journal of endocrinology 144 (1995) 49-59.

[50] E. Bajetta, L. Ferrari, A. Martinetti, L. Celio, G. Procopio, S. Artale, N. Zilembo, M. Di Bartolomeo, E. Seregni, E. Bombardieri, Chromogranin A, neuron specific enolase, carcinoembryonic antigen, and hydroxyindole acetic acid evaluation in patients with neuroendocrine tumors, Cancer 86 (1999) 858-865.

[51] E. Baudin, A. Gigliotti, M. Ducreux, J. Ropers, E. Comoy, J.C. Sabourin, J.M. Bidart, A.F. Cailleux, R. Bonacci, P. Ruffie, M. Schlumberger, Neuron-specific enolase and chromogranin A as markers of neuroendocrine tumours, Br. J. Cancer 78 (1998) 1102-1107.

[52] J.A. Norton, M.L. Melcher, F. Gibril, R.T. Jensen, Gastric carcinoid tumors in multiple endocrine neoplasia-1 patients with Zollinger-Ellison syndrome can be symptomatic, demonstrate aggressive growth, and require surgical treatment, Surgery 136 (2004) 1267-1274.

[53] A. Sundin, M.P. Vullierme, G. Kaltsas, U. Plockinger, ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Tumors: radiological examinations, Neuroendocrinology 90 (2009) 167-183.

[54] A. Sundin, U. Garske, H. Orlefors, Nuclear imaging of neuroendocrine tumours, Best practice & research. Clinical endocrinology & metabolism 21 (2007) 69-85.

[55] S.W. Lamberts, W.W. de Herder, L.J. Hofland, Somatostatin analogs in the diagnosis and treatment of cancer, Trends in endocrinology and metabolism: TEM 13 (2002) 451-457.

[56] L.J. Hofland, S.W. Lamberts, The pathophysiological consequences of somatostatin receptor internalization and resistance, Endocr Rev 24 (2003) 28-47.

[57] J.S. Lewis, A. Srinivasan, M.A. Schmidt, C.J. Anderson, In vitro and in vivo evaluation of 64Cu-TETA-Tyr3-octreotate. A new somatostatin analog with improved target tissue uptake, Nucl. Med. Biol. 26 (1999) 267-273.

[58] M. Schottelius, T. Poethko, M. Herz, J.C. Reubi, H. Kessler, M. Schwaiger, H.J. Wester, First (18)F-labeled tracer suitable for routine clinical imaging of sst receptor-expressing tumors using positron emission tomography, Clinical cancer research : an official journal of the American Association for Cancer Research 10 (2004) 3593-3606.

[59] S. Fanti, V. Ambrosini, P. Tomassetti, P. Castellucci, G. Montini, V. Allegri, G. Grassetto, D. Rubello, C. Nanni, R. Franchi, Evaluation of unusual neuroendocrine tumours by means of 68Ga-DOTA-NOC PET, Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 62 (2008) 667-671.

[60] M. Gabriel, C. Decristoforo, D. Kendler, G. Dobrozemsky, D. Heute, C. Uprimny, P. Kovacs, E. Von Guggenberg, R. Bale, I.J. Virgolini, 68Ga-DOTA-Tyr3-octreotide PET in neuroendocrine tumors: comparison with somatostatin receptor scintigraphy and CT, Journal of nuclear medicine : official publication, Society of Nuclear Medicine 48 (2007) 508-518.

[61] V. Ambrosini, P. Tomassetti, P. Castellucci, D. Campana, G. Montini, D. Rubello, C. Nanni, A. Rizzello, R. Franchi, S. Fanti, Comparison between 68Ga-DOTA-NOC and 18F-DOPA PET for the detection of gastro-entero-pancreatic and lung neuro-endocrine tumours, European journal of nuclear medicine and molecular imaging 35 (2008) 1431-1438.

[62] K.P. Koopmans, O.N. Neels, I.P. Kema, P.H. Elsinga, T.P. Links, E.G. de Vries, P.L. Jager, Molecular imaging in neuroendocrine tumors: molecular uptake mechanisms and clinical results, Crit. Rev. Oncol. Hematol. 71 (2009) 199-213.

[63] J.D. Erickson, M.K. Schafer, T.I. Bonner, L.E. Eiden, E. Weihe, Distinct pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human vesicular monoamine transporter, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5166-5171.

[64] F. Verrey, System L: heteromeric exchangers of large, neutral amino acids involved in directional transport, Pflugers Archiv : European journal of physiology 445 (2003) 529-533.

[65] G. Firnau, S. Sood, R. Chirakal, C. Nahmias, E.S. Garnett, Metabolites of 6-[18F]fluoro-L-dopa in human blood, Journal of nuclear medicine : official publication, Society of Nuclear Medicine 29 (1988) 363-369.

[66] P. Soares-da-Silva, M.P. Serrao, High- and low-affinity transport of L-leucine and L-DOPA by the hetero amino acid exchangers LAT1 and LAT2 in LLC-PK1 renal cells, American journal of physiology. Renal physiology 287 (2004) F252-261.

[67] S. Hoegerle, C. Altehoefer, N. Ghanem, G. Koehler, C.F. Waller, H. Scheruebl, E. Moser, E. Nitzsche, Whole-body 18F dopa PET for detection of gastrointestinal carcinoid tumors, Radiology 220 (2001) 373-380.

[68] A. Becherer, M. Szabo, G. Karanikas, P. Wunderbaldinger, P. Angelberger, M. Raderer, A. Kurtaran, R. Dudczak, K. Kletter, Imaging of advanced neuroendocrine tumors with (18)F-FDOPA PET, Journal of nuclear medicine : official publication, Society of Nuclear Medicine 45 (2004) 1161-1167.

[69] K.P. Koopmans, E.G. de Vries, I.P. Kema, P.H. Elsinga, O.C. Neels, W.J. Sluiter, A.N. van der Horst-Schrivers, P.L. Jager, Staging of carcinoid tumours with 18F-DOPA PET: a prospective, diagnostic accuracy study, Lancet Oncol 7 (2006) 728-734.

[70] N. Furuta, H. Kiyota, F. Yoshigoe, N. Hasegawa, Y. Ohishi, Diagnosis of pheochromocytoma using [123I]-compared with [131I]-metaiodobenzylguanidine scintigraphy, International journal of urology : official journal of the Japanese Urological Association 6 (1999) 119-124.

[71] P. Ruszniewski, G. Delle Fave, G. Cadiot, P. Komminoth, D. Chung, B. Kos-Kudla, R. Kianmanesh, D. Hochhauser, R. Arnold, H. Ahlman, S. Pauwels, D.J. Kwekkeboom, G. Rindi, Well-differentiated gastric tumors/carcinomas, Neuroendocrinology 84 (2006) 158-164.

[72] N.C.C.N. (NCCN), Practice Guidelines in Oncology (http://www.NCCN.org), in, 2014.

[73] I.M. Modlin, K.D. Lye, M. Kidd, A 5-decade analysis of 13,715 carcinoid tumors, Cancer 97 (2003) 934-959.

[74] C. Makridis, K. Oberg, C. Juhlin, J. Rastad, H. Johansson, L.E. Lorelius, G. Akerstrom, Surgical treatment of mid-gut carcinoid tumors, World J. Surg. 14 (1990) 377-383; discussion 384-375.

[75] M.L. McGory, M.A. Maggard, H. Kang, J.B. O'Connell, C.Y. Ko, Malignancies of the appendix: beyond case series reports, Dis. Colon Rectum 48 (2005) 2264-2271.

[76] B.N. Fahy, L.H. Tang, D. Klimstra, W.D. Wong, J.G. Guillem, P.B. Paty, L.K. Temple, J. Shia, M.R. Weiser, Carcinoid of the rectum risk stratification (CaRRS): a strategy for preoperative outcome assessment, Ann. Surg. Oncol. 14 (2007) 396-404.

[77] O.N. Tucker, P.L. Crotty, K.C. Conlon, The management of insulinoma, The British journal of surgery 93 (2006) 264-275.

[78] P. Hellman, M. Andersson, J. Rastad, C. Juhlin, S. Karacagil, B. Eriksson, B. Skogseid, G. Akerstrom, Surgical strategy for large or malignant endocrine pancreatic tumors, World J. Surg. 24 (2000) 1353-1360.

[79] M. Falconi, U. Plockinger, D.J. Kwekkeboom, R. Manfredi, M. Korner, L. Kvols, U.F. Pape, J. Ricke, P.E. Goretzki, S. Wildi, T. Steinmuller, K. Oberg, J.Y. Scoazec, Well-differentiated pancreatic nonfunctioning tumors/carcinoma, Neuroendocrinology 84 (2006) 196-211.

[80] C.C. Solorzano, J.E. Lee, P.W. Pisters, J.N. Vauthey, G.D. Ayers, M.E. Jean, R.F. Gagel, J.A. Ajani, R.A. Wolff, D.B. Evans, Nonfunctioning islet cell carcinoma of the pancreas: survival results in a contemporary series of 163 patients, Surgery 130 (2001) 1078-1085.

[81] J.A. Norton, M. Kivlen, M. Li, D. Schneider, T. Chuter, R.T. Jensen, Morbidity and mortality of aggressive resection in patients with advanced neuroendocrine tumors, Arch. Surg. 138 (2003) 859-866.

[82] B. Eriksson, New drugs in neuroendocrine tumors: rising of new therapeutic philosophies?, Curr. Opin. Oncol. 22 (2010) 381-386.

[83] S. Faivre, M.P. Sablin, C. Dreyer, E. Raymond, Novel anticancer agents in clinical trials for well-differentiated neuroendocrine tumors, Endocrinol. Metab. Clin. North Am. 39 (2010) 811-826.

[84] A. Rinke, H.H. Muller, C. Schade-Brittinger, K.J. Klose, P. Barth, M. Wied, C. Mayer, B. Aminossadati, U.F. Pape, M. Blaker, J. Harder, C. Arnold, T. Gress, R. Arnold, Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: a report from the PROMID Study Group, J Clin Oncol 27 (2009) 4656-4663.

[85] I.M. Modlin, M. Pavel, M. Kidd, B.I. Gustafsson, Review article: somatostatin analogues in the treatment of gastroenteropancreatic neuroendocrine (carcinoid) tumours, Aliment. Pharmacol. Ther. 31 (2010) 169-188.

[86] K.Y. Gulenchyn, X. Yao, S.L. Asa, S. Singh, C. Law, Radionuclide therapy in neuroendocrine tumours: a systematic review, Clin. Oncol. 24 (2012) 294-308.

[87] G. Kaltsas, A. Rockall, D. Papadogias, R. Reznek, A.B. Grossman, Recent advances in radiological and radionuclide imaging and therapy of neuroendocrine tumours, Eur. J. Endocrinol. 151 (2004) 15-27.

[88] D.J. Kwekkeboom, W.W. de Herder, B.L. Kam, C.H. van Eijck, M. van Essen, P.P. Kooij, R.A. Feelders, M.O. van Aken, E.P. Krenning, Treatment with the radiolabeled somatostatin

analog [177 Lu-DOTA 0,Tyr3]octreotate: toxicity, efficacy, and survival, J Clin Oncol 26 (2008) 2124-2130.

[89] D.L. Bushnell, Jr., T.M. O'Dorisio, M.S. O'Dorisio, Y. Menda, R.J. Hicks, E. Van Cutsem, J.L. Baulieu, F. Borson-Chazot, L. Anthony, A.B. Benson, K. Oberg, A.B. Grossman, M. Connolly, H. Bouterfa, Y. Li, K.A. Kacena, N. LaFrance, S.A. Pauwels, 90Y-edotreotide for metastatic carcinoid refractory to octreotide, J Clin Oncol 28 (2010) 1652-1659.

[90] D. O'Toole, O. Hentic, O. Corcos, P. Ruszniewski, Chemotherapy for gastro-enteropancreatic endocrine tumours, Neuroendocrinology 80 Suppl 1 (2004) 79-84.

[91] L.E. Broder, S.K. Carter, Pancreatic islet cell carcinoma. II. Results of therapy with streptozotocin in 52 patients, Ann. Intern. Med. 79 (1973) 108-118.

[92] C.G. Moertel, J.A. Hanley, L.A. Johnson, Streptozocin alone compared with streptozocin plus fluorouracil in the treatment of advanced islet-cell carcinoma, The New England journal of medicine 303 (1980) 1189-1194.

[93] C.G. Moertel, M. Lefkopoulo, S. Lipsitz, R.G. Hahn, D. Klaassen, Streptozocin-doxorubicin, streptozocin-fluorouracil or chlorozotocin in the treatment of advanced islet-cell carcinoma, The New England journal of medicine 326 (1992) 519-523.

[94] M.A. Kouvaraki, J.A. Ajani, P. Hoff, R. Wolff, D.B. Evans, R. Lozano, J.C. Yao, Fluorouracil, doxorubicin, and streptozocin in the treatment of patients with locally advanced and metastatic pancreatic endocrine carcinomas, J Clin Oncol 22 (2004) 4762-4771.

[95] M.H. Kulke, K. Stuart, P.C. Enzinger, D.P. Ryan, J.W. Clark, A. Muzikansky, M. Vincitore, A. Michelini, C.S. Fuchs, Phase II study of temozolomide and thalidomide in patients with metastatic neuroendocrine tumors, J Clin Oncol 24 (2006) 401-406.

[96] S. De Dosso, E. Grande, J. Barriuso, D. Castellano, J. Tabernero, J. Capdevila, The targeted therapy revolution in neuroendocrine tumors: in search of biomarkers for patient selection and response evaluation, Cancer metastasis reviews 32 (2013) 465-477.

[97] A. Couvelard, D. O'Toole, H. Turley, R. Leek, A. Sauvanet, C. Degott, P. Ruszniewski, J. Belghiti, A.L. Harris, K. Gatter, F. Pezzella, Microvascular density and hypoxia-inducible factor pathway in pancreatic endocrine tumours: negative correlation of microvascular density and VEGF expression with tumour progression, Br. J. Cancer 92 (2005) 94-101.

[98] E. Raymond, L. Dahan, J.L. Raoul, Y.J. Bang, I. Borbath, C. Lombard-Bohas, J. Valle, P. Metrakos, D. Smith, A. Vinik, J.S. Chen, D. Horsch, P. Hammel, B. Wiedenmann, E. Van Cutsem, S. Patyna, D.R. Lu, C. Blanckmeister, R. Chao, P. Ruszniewski, Sunitinib malate for the treatment of pancreatic neuroendocrine tumors, The New England journal of medicine 364 (2011) 501-513.

[99] R.D. Beauchamp, R.J. Coffey, Jr., R.M. Lyons, E.A. Perkett, C.M. Townsend, Jr., H.L. Moses, Human carcinoid cell production of paracrine growth factors that can stimulate fibroblast and endothelial cell growth, Cancer Res. 51 (1991) 5253-5260.

[100] A. Chaudhry, K. Funa, K. Oberg, Expression of growth factor peptides and their receptors in neuroendocrine tumors of the digestive system, Acta Oncol. 32 (1993) 107-114.

[101] C.H. Heldin, Targeting the PDGF signaling pathway in tumor treatment, Cell communication and signaling : CCS 11 (2013) 97.

[102] H. Frucht, J.M. Howard, J.I. Slaff, S.A. Wank, D.M. McCarthy, P.N. Maton, R. Vinayek, J.D. Gardner, R.T. Jensen, Secretin and calcium provocative tests in the Zollinger-Ellison syndrome. A prospective study, Ann. Intern. Med. 111 (1989) 713-722.

[103] H. Konno, T. Arai, T. Tanaka, M. Baba, K. Matsumoto, T. Kanai, S. Nakamura, S. Baba, Y. Naito, H. Sugimura, A. Yukita, M. Asano, H. Suzuki, Antitumor effect of a neutralizing antibody to vascular endothelial growth factor on liver metastasis of endocrine neoplasm, Japanese journal of cancer research : Gann 89 (1998) 933-939.

[104] K.W. Liu, B. Hu, S.Y. Cheng, Platelet-derived growth factor signaling in human malignancies, Chinese journal of cancer 30 (2011) 581-584.

[105] K.W. Liu, H. Feng, R. Bachoo, A. Kazlauskas, E.M. Smith, K. Symes, R.L. Hamilton, M. Nagane, R. Nishikawa, B. Hu, S.Y. Cheng, SHP-2/PTPN11 mediates gliomagenesis driven by PDGFRA and INK4A/ARF aberrations in mice and humans, The Journal of clinical investigation 121 (2011) 905-917.

[106] J. Andrae, R. Gallini, C. Betsholtz, Role of platelet-derived growth factors in physiology and medicine, Genes Dev. 22 (2008) 1276-1312.

[107] J. Zhang, P. Wang, M. Dykstra, P. Gelebart, D. Williams, R. Ingham, E.E. Adewuyi, R. Lai, T. McMullen, Platelet-derived growth factor receptor-alpha promotes lymphatic metastases in papillary thyroid cancer, The Journal of pathology 228 (2012) 241-250.

[108] N.R. Wall, Y. Shi, Small RNA: can RNA interference be exploited for therapy?, Lancet 362 (2003) 1401-1403.

[109] J. Barretina, G. Caponigro, N. Stransky, K. Venkatesan, A.A. Margolin, S. Kim, C.J. Wilson, J. Lehar, G.V. Kryukov, D. Sonkin, A. Reddy, M. Liu, L. Murray, M.F. Berger, J.E. Monahan, P. Morais, J. Meltzer, A. Korejwa, J. Jane-Valbuena, F.A. Mapa, J. Thibault, E. Bric-Furlong, P. Raman, A. Shipway, I.H. Engels, J. Cheng, G.K. Yu, J. Yu, P. Aspesi, Jr., M. de Silva, K. Jagtap, M.D. Jones, L. Wang, C. Hatton, E. Palescandolo, S. Gupta, S. Mahan, C. Sougnez, R.C. Onofrio, T. Liefeld, L. MacConaill, W. Winckler, M. Reich, N. Li, J.P. Mesirov, S.B. Gabriel, G. Getz, K. Ardlie, V. Chan, V.E. Myer, B.L. Weber, J. Porter, M. Warmuth, P. Finan, J.L. Harris, M. Meyerson, T.R. Golub, M.P. Morrissey, W.R. Sellers, R. Schlegel, L.A. Garraway, The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity, Nature 483 (2012) 603-607.

[110] Z. von Marschall, A. Scholz, T. Cramer, G. Schafer, M. Schirner, K. Oberg, B. Wiedenmann, M. Hocker, S. Rosewicz, Effects of interferon alpha on vascular endothelial growth factor gene transcription and tumor angiogenesis, J. Natl. Cancer Inst. 95 (2003) 437-448.

[111] F. Pampaloni, E.G. Reynaud, E.H. Stelzer, The third dimension bridges the gap between cell culture and live tissue, Nature reviews. Molecular cell biology 8 (2007) 839-845.

[112] J.H. Tsai, J. Yang, Epithelial-mesenchymal plasticity in carcinoma metastasis, Genes Dev. 27 (2013) 2192-2206.

[113] R. Kalluri, R.A. Weinberg, The basics of epithelial-mesenchymal transition, The Journal of clinical investigation 119 (2009) 1420-1428.

[114] M.L. Gild, M. Bullock, B.G. Robinson, R. Clifton-Bligh, Multikinase inhibitors: a new option for the treatment of thyroid cancer, Nature reviews. Endocrinology 7 (2011) 617-624.

[115] K. Okada, K. Komuta, S. Hashimoto, S. Matsuzaki, T. Kanematsu, T. Koji, Frequency of apoptosis of tumor-infiltrating lymphocytes induced by fas counterattack in human colorectal carcinoma and its correlation with prognosis, Clinical cancer research : an official journal of the American Association for Cancer Research 6 (2000) 3560-3564.

[116] K.M. Hajra, D.Y. Chen, E.R. Fearon, The SLUG zinc-finger protein represses E-cadherin in breast cancer, Cancer Res. 62 (2002) 1613-1618.

[117] J. Comijn, G. Berx, P. Vermassen, K. Verschueren, L. van Grunsven, E. Bruyneel, M. Mareel, D. Huylebroeck, F. van Roy, The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion, Mol. Cell 7 (2001) 1267-1278.

[118] J. Yang, S.A. Mani, J.L. Donaher, S. Ramaswamy, R.A. Itzykson, C. Come, P. Savagner, I. Gitelman, A. Richardson, R.A. Weinberg, Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis, Cell 117 (2004) 927-939.

[119] M.A. Perez-Moreno, A. Locascio, I. Rodrigo, G. Dhondt, F. Portillo, M.A. Nieto, A. Cano, A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions, The Journal of biological chemistry 276 (2001) 27424-27431.

[120] E. Batlle, E. Sancho, C. Franci, D. Dominguez, M. Monfar, J. Baulida, A. Garcia De Herreros, The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells, Nature cell biology 2 (2000) 84-89.

[121] A. Cano, M.A. Perez-Moreno, I. Rodrigo, A. Locascio, M.J. Blanco, M.G. del Barrio, F. Portillo, M.A. Nieto, The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression, Nature cell biology 2 (2000) 76-83.

[122] M. Jechlinger, A. Sommer, R. Moriggl, P. Seither, N. Kraut, P. Capodiecci, M. Donovan, C. Cordon-Cardo, H. Beug, S. Grunert, Autocrine PDGFR signaling promotes mammary cancer metastasis, The Journal of clinical investigation 116 (2006) 1561-1570.

[123] M.G. Benesch, Y.M. Ko, T.P. McMullen, D.N. Brindley, Autotaxin in the crosshairs: taking aim at cancer and other inflammatory conditions, FEBS Lett. 588 (2014) 2712-2727.

[124] J. So, F.Q. Wang, J. Navari, J. Schreher, D.A. Fishman, LPA-induced epithelial ovarian cancer (EOC) in vitro invasion and migration are mediated by VEGF receptor-2 (VEGF-R2), Gynecol. Oncol. 97 (2005) 870-878.

[125] M.M. Murph, J. Hurst-Kennedy, V. Newton, D.N. Brindley, H. Radhakrishna, Lysophosphatidic acid decreases the nuclear localization and cellular abundance of the p53 tumor suppressor in A549 lung carcinoma cells, Molecular cancer research : MCR 5 (2007) 1201-1211.

[126] S. Liu, M. Umezu-Goto, M. Murph, Y. Lu, W. Liu, F. Zhang, S. Yu, L.C. Stephens, X. Cui, G. Murrow, K. Coombes, W. Muller, M.C. Hung, C.M. Perou, A.V. Lee, X. Fang, G.B. Mills, Expression of autotaxin and lysophosphatidic acid receptors increases mammary tumorigenesis, invasion, and metastases, Cancer cell 15 (2009) 539-550.

[127] E.C. LaCasse, Pulling the plug on a cancer cell by eliminating XIAP with AEG35156, Cancer Lett. 332 (2013) 215-224.

[128] S. Fulda, D. Vucic, Targeting IAP proteins for therapeutic intervention in cancer, Nature reviews. Drug discovery 11 (2012) 109-124.

[129] H. Kashkar, X-linked inhibitor of apoptosis: a chemoresistance factor or a hollow promise, Clinical cancer research : an official journal of the American Association for Cancer Research 16 (2010) 4496-4502.

[130] P.H. Krammer, CD95's deadly mission in the immune system, Nature 407 (2000) 789-795.

[131] J. O'Connell, G.C. O'Sullivan, J.K. Collins, F. Shanahan, The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand, The Journal of experimental medicine 184 (1996) 1075-1082.

[132] E. Song, J. Chen, N. Ouyang, F. Su, M. Wang, U. Heemann, Soluble Fas ligand released by colon adenocarcinoma cells induces host lymphocyte apoptosis: an active mode of immune evasion in colon cancer, Br. J. Cancer 85 (2001) 1047-1054.

[133] M.W. Bennett, J. O'Connell, G.C. O'Sullivan, C. Brady, D. Roche, J.K. Collins, F. Shanahan, The Fas counterattack in vivo: apoptotic depletion of tumor-infiltrating lymphocytes associated with Fas ligand expression by human esophageal carcinoma, J. Immunol. 160 (1998) 5669-5675.