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EGFR/TGFα and TGFβ/CTGF Signaling in Neuroendocrine Neoplasia: Theoretical Therapeutic Targets

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Abstract

Neuroendocrine neoplasms (NENs) are a heterogeneous family of malignancies whose proliferation is partially dependent on growth factors secreted by the microenvironment and the tumor itself. Growth factors of demonstrated importance in experimental models of NENs include EGF, TGFα, TGFβ and CTGF. EGF and TGFα bind to EGFR to stimulate an intact RAS/RAF/MAPK pathway, leading to transcription of genes associated with cell proliferation, invasion and metastasis. Theoretically, TGFα stimulation can be inhibited at several points of the MAPK pathway, but success is limited to NEN models and is not evident in the clinical setting. TGFβ1 stimulates TGFβRI and II resulting neuroendocrine cell growth inhibition through SMAD-mediated activation of the growth inhibitor $P21^{(WAF1/CIP1)}$. Although some NENs are inhibited by TGFβ1, paradoxical growth is seen in experimental models of gastric and small intestinal NENs. Therapeutic targeting of TGFβ1 in NENs is therefore complicated by uncertainty regarding the direction of proliferative regulation accorded by TGFβ1 secretion. CTGF expression is associated with more malignant clinical phenotypes in a variety of cancers including NENs. CTGF promotes growth in gastric and small intestinal NEN models, and is implicated as a mediator of local and distant fibrosis caused by NENs of enterochromaffin cell origin. CTGF inhibitors are available but untried in NENs for their anti-proliferative effect. In summary, growth factors are essential to NEN proliferation, and although intervention targeting these proteins is effective in experimental models, limited clinical efficacy has been identified.

Introduction

Although Gastroenteropancreatic (GEP) and bronchopulmonary (BP) neuroendocrine neoplasms (NENs) comprise ~2% of all malignancies[1], they are rapidly increasing in incidence and prevalence. These cancers exhibit a wide spectrum of biological and malignant phenotypes and range from neoplasms that are largely “benign”, such as gastrin-responsive gastric ECL cell neoplasms (Type 1 gastric “carcinoids”) or pancreatic β-cell tumors, to aggressive tumors with a poor prognosis such as pancreatic neuroendocrine cancers (NECs), colon NECs and small cell lung cancers (SCLC) [1, 2]. Apart from gastric NENs that are driven by the hypergastrinemia associated with a low acid state, the etiopathogenesis of NENs is unknown. They are considered to evolve from DNA damaging events to neuroendocrine committed stem cells while a minority are associated with known inherited menin-related genetic events [1, 3].

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Overall, this class of neoplasia has not been well-studied and consequently few targeted therapies exist apart from agents that activate somatostatin receptors [4, 5]. More recently, other receptors and regulatory pathways have been identified (tyrosine kinases, VEGFR, PGFDR, mTOR) that appear to have some utility as therapeutic targets. For example, both, the tyrosine kinase inhibitor Sunitinib and the mTOR inhibitor Everolimus have been shown to improve progression free survival in patients with advanced pancreatic NENs although substantial concerns have been raised by rigorous critics of the design of both studies [6, 7]. In this review, we examine the EGFR/TGFα, TGFβ and CTGF class of receptors and growth factors as proliferative regulators in GEP- and BP-NENs. The overview focuses on transcriptional status and expression levels, chromosomal or mutational alterations and signal pathway activation related to NENs. In addition, we describe the biological mechanisms by which NENs modify their microenvironment (peritoneal fibrosis or valvular fibrosis, hepatic metastases) and the clinical implications of targeted therapy for this class of growth factors.

EGF/TGFα, EGFR Signaling: Candidate, Multilevel Targets

Epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) are polypeptides that bind the EGF receptors activating signal transduction pathways (RAS-RAF-MAPK) that regulate cellular responses to growth signals.

EGF is a 53 amino-acid polypeptide (6kDA protein) derived from a large precursor molecule, proteolytically cleaved to generate the final peptide. EGF is encoded on chromosome 4q25; alternate splicing results in multiple transcript variants. Biologically, EGF is a mitogenic factor regulating growth, proliferation and differentiation of numerous cell types; abnormalities in EGF-signaling pathways have been associated with the growth and progression of neoplasia [8].

TGFα is a related growth factor to EGF and regulates the same signaling pathways through activation of the same receptors. Co-expression of this 50 amino-acid polypeptide and EGFR confers growth advantage to tumor cells [9]. TGFα is expressed in ~70–100% of NENs depending on the technique used (immunohistochemistry or northern analysis) [9–12], and is typically over-expressed in larger rectal NENs with a high proliferative index (as measured by Ki-67) [12]. Co-expression of TGFα and EGFR occurs in ~80% of these neoplasms [12].

EGF and TGFα bind to high affinity cell surface receptors (EGFR), which are receptor tyrosine kinase members of the ErbB family. Both growth factors specifically bind to the extracellular ligand binding domain of HER1 (ErbB1) and signaling is initiated following receptor homo-/hetero-dimerization and autophosphorylation by the intracellular kinase domain (fig. 1). Phosphorylation of cytoplasmic substrates occurs and initiates a signaling cascade (RAS/RAF/MAPK-ERK) that drives pro-proliferative gene expression, cytoskeletal rearrangement, and increased cell proliferation [8]. Somatic mutations in the tyrosine kinase domain of HER1 result in constitutive kinase activity and unregulated pro-proliferation signaling. Such tumors are typically susceptible to tyrosine kinase inhibitors (TKIs) [13].

Expression of HER-1 (EGFR) and HER-4 have been indentified in NENs. Immunohistochemical and *in situ* studies identify HER1 in the majority (80–100%) of small intestinal and rectal NENs [10–12] but it is unclear whether the intracellular domain is expressed [9]. HER-4 was expressed in ~90% of the cases in a mixed group of NENs [14], but its role in tumorigenesis remains unclear. In GEP-NENs, EGFR (HER1) is expressed in gastrinomas [11]. Expression of EGFR is frequent (~60%) in all lung NENs [15] although SCLCs tend to exhibit low levels of EGFR [16]. At a molecular level, GEP-NENs express EGFR aneusomy (20% of cases) and elevated EGFR copy number (39%) [17]. These results

suggest that NENs exhibit an intact, pro-proliferative pathway that may regulate tumor growth and be potentially targetable.

A well-described target is the tyrosine kinase domain which can be inhibited by agents such as Erlotinib or Gefitinib (table 1). These are first-generation agents which have only been show to be effective in treating lung cancer in individuals that express mutations in EGFR (response rates of 40–71% in mutation-positive tumors versus 1.1% in EGFR mutation-negative) [18]. Activating somatic mutations are usually present in exons 18, 19, and 21 of HER1. However, in a PCR assessment of 31 BP-NENs, no mutations in the *EGFR* kinase domain, that were predictive of a response to EGFR TKIs, were detected [19]. A similar finding was noted in 102 GEP-NENs [17]. EGFR TK mutations also preferentially activate antiapoptotic pathways (e.g. PI3K/AKT) [20] suggesting TKIs and AKT inhibitors may be useful under these conditions. However, TK mutations are uncommon in NENs which suggests these tumors may not be candidates for this class of agents. A phase II study of Gefitinib that included 57 patients with GEP-NENs demonstrated that only one of 40 evaluable patients achieved a radiological response; however, 32% had an increased time to progression [21]. The low efficacy highlights the absence of facilitating EGFR tyrosine kinase mutations associated with Gefitinib activity. This also suggests a combinatorial approach of TKIs and AKT/mTOR inhibitors is unlikely to be efficacious.

SCLC cell lines indicate that targeting these very aggressive neoplasms with Gefitinib may be potentially effective as MAPK signaling could be inhibited [16]. However, similar to GEP-NENs, facilitating EGFR mutations are rare in these tumors (<3%) [22] indicating current EGFR TKIs may be ineffective. An alternative mechanism for reducing growth-signaling in these neoplasms has been identified by evaluating antagonists of growth hormone releasing hormone and bombesin have since these agents inhibit the expression of EGF/HER receptor family in SCLC cell lines [23]. Therefore, although EGFR remains a rational target, and has been successfully targeted using monoclonal antibodies e.g., cetuximab in colorectal cancer [24], little is known regarding the therapeutic potential of targeting this receptor in NENs. One caveat with targeting this receptor is the potential for cross-activation of the PI3K/AKT/mTOR pathway, a pro-proliferative pathway that tumor cells may activate when EGFR signaling is inhibited [25].

The EGFR-mediated RAS/RAF/MAPK signaling pathway is usually activated in NENs [26, 27]. Immunohistochemistry demonstrates expression of the BRAF-activator, Rap1, as well as B-Raf itself in GEP-NENs [28], suggesting that one component of EGFR-signaling, BRAF itself, might be a candidate target. BRAF, however, is not mutated in either GEP- or BP-NENs [26, 29]. In the neuroendocrine cell lines, BON (lymph node metastasis of a pancreatic NEN) and INS-1 (a rat insulinoma cell line), over expression of Rap1 and B-Raf activated MAPK ERK-2 and ERK-dependent transcription factor Elk-1 has been noted [28]. BAY43-9006 (Sorafenib), which suppresses BRAF activity, inhibited growth and induced apoptosis in these cells and suppressed phosphorylation of MAPK ERK1/2 and its upstream kinase MEK1/2 suggesting targeting EGFR signaling may show some promise in pancreatic-derived NENs [28] (table 1). In non-pancreatic NENs, mutations in KRAS, upstream of MAPK have been identified in three NENs (of 102 samples) [17]. All mutations were heterozygous and two of them were associated with a non-response to anti-EGFR monoclonal antibodies. The relevance of this to current therapeutic strategies is unclear.

Short-term cultured small intestinal NENs secrete detectable TGFα (400–700 pM), which can be inhibited by targeting sst2 [10]. Exogenous TGFα stimulated growth of these neoplasms *in vitro* which could be partially blocked by the use of neutralizing anti-EGF receptor monoclonal antibodies [10] suggesting an autocrine-mediated growth regulatory loop. Other evidence for proliferative roles for these factors comes from studies with BON and

KRJ-I (small intestine EC cell-derived NEN cell line) [30]. Both EGF and TGFα (EC_{50} = 15.8 and 10 ng/ml) stimulate BON proliferation, while only TGFα (EC_{50} = 0.63 ng/ml) stimulates KRJ-I proliferation [31]. Targeting the stress-responsive molecular chaperone, Hsp90, with 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) reduces EGFR expression in a lung cell line (NCI-H727) [17] providing a further potential avenue for investigation.

In the *Mastomys* animal model of gastric NENs (ECL cell tumors), the proliferative effect of TGFα on ECL cells is specifically amplified during the development of gastric mucosal hyperplasia [32] when TGFα and EGFR expression increased in transforming gastric ECL cells [32]. This suggests that during low acid-induced hypergastrinemia, expression of TGFα and EGFR may constitute an autocrine regulatory mechanism in ECL cell tumor transformation.

In the RIP1-Tag2 (RT2) mouse model of pancreatic NENs, EGFR inhibitor treatment (erlotinib) resulted in a reduced growth rate of tumors with no abnormalities in EGFR [33]. This was associated with increased apoptosis and reduced neovascularization. These effects appeared to be mediated by TGFα (apoptosis) and HB-EGF (angiogenesis) (through signaling in the tumor microenvironment) suggesting more dynamic roles for a non-mutated EGF receptor in NEN pathogenesis.

These data suggest that proliferation in both GEP- and BP-NENs is stimulated by growth-regulatory factors such as EGF and TGFα which function through an intact EGFR to regulate RAS/RAF/MAPK signaling pathway. The source of TGFα appears to be autocrine. Despite making an attractive therapeutic target, at multiple levels, there is no currently available agent in clinical use for NENs.

The TGFβ1 Superfamily: Positive and Negative Roles in Proliferation

The TGFβ superfamily encodes a range of secreted protein including TGFβ1, TGFβ2 and TGFβ3 as well as inhibins and Bone Morphogenic Proteins (BMPs) [34]. TGFβs are effective and ubiquitous mediators of cell growth via interaction with TGFβ receptor I and II (TGFβRI and TGFβRII). They are considered potent inhibitors of normal cell growth in the physiological setting, but cells undergoing malignant transformation become either partly or completely resistant to TGFβ growth inhibition. In fact, there is growing evidence that during the later stages of cancer development, TGFβ has a paradoxical pro-proliferative effect. TGFβ is actively secreted by tumor cells and contributes to cell growth, invasion, and metastasis while decreasing host-tumor immune response [35]. The majority of work in NENs has focused on TGFβ1.

TGFβ1, which is encoded on Chr19q13.1, is a 44.3kDa protein that is usually secreted as in an inactive form consisting of a homodimer non-covalently linked to a latency-associated peptide (LAP) homodimer. Additional processing to release the active form involves MMPs, alterations in pH, production or ROS or the activity of thrombospondin-1 [34]. The active TGFβ1 binds to type II TGFβ receptors which form heterodimers with type I receptors. This results in receptor-mediated serine-threonine kinase activity with phosphorylation of the SMAD family of transcription factors and activation/inhibition of various genes, depending on the state of cell transformation (fig. 2) [34, 35].

At a signaling pathway level, NENs express SMADs (SMAD2,3,4), but no SMAD4 mutations have been identified in small intestinal NENs [36]. In addition, SMAD3 has not been identified as a tumor suppressor; although loss of heterozygosity has been noted for markers in ~20% of NENs, no acquired clonal mutations, insertions, or microdeletions in SMAD3 were detected [37]. NENs exhibit variable expression of the TGFβ1 cytostatic

program target protein P21(WAF1/CIP1) [38, 39] and also frequently express c-Myc [40], a TGFβ1 pathway antagonist.

Immunohistochemical studies identify TGFβ1 and its receptors in the majority of GEP-NENs [11, 41–43] suggesting these lesions may be a biological target for paracrine and autocrine antimitogenic actions of this growth factor. It should be noted that lesions exhibit variable expression of TGFβRII [44], but do not exhibit microsatellite instabilities (leading to early termination) in this receptor.

Three studies have examined the role of TGFβ in NEN cell lines. In BON cells, TGFβ1 treatment resulted in transactivation of a TGFβ–responsive reporter construct as well as inhibition of c-Myc and induction of $P21^{WAF1/CIP1}$ expression [42]. TGFβ1 also inhibited anchorage-dependent and independent growth in a time and dose dependent manner [42], leading to G1 growth arrest without evidence of apoptosis [42]. Functional inactivation of endogenous TGFβ1 revealed the existence of an autocrine antiproliferative loop in BON cells.

In another study, comparing normal small intestinal EC cells with KRJ-I cells, the growth of normal cells could be inhibited by TGFβ1 while KRJ-I cells lost this TGFβ1-mediated cytostasis and were induced to proliferate by TGFB1[45]. Additional examination of the TGFβ1 pathway demonstrated low expression levels (mRNA and protein) of non-mutated TGFβRII, phosphorylatable SMAD2 (indicating an intact TGFβ1:TGFβRII signaling) but absent nuclear targeting of pSMAD2. These data suggest that TGFβ1-mediated signal transduction in this cell line, as in glioma cell lines [46], is blocked at the level of SMAD nuclear translocation. This phenomenon was specifically associated with increased expression of the inhibitor of SMAD nuclear translocation, SMAD7, down-regulated $p21^{WAF1/CIP1}$ transcription and increased expression of c-Myc as well as phosphorylation and cross-activation of ERK1/2 as well as downstream activation of the malignancy-defining genes *MTA1* with loss of *E-cadherin*. These studies identify that small intestinal NENs, unlike pancreatic NENs do not express a TGFβ1-mediated autocrine antiproliferative loop and that this growth factor may be involved in regulating the metastatic phenotype [45].

In two other studies, TGFβRII was examined in SCLC cell lines and in 80–100% identified to be expressed at low levels [47, 48]. This lack or absence of TGFβRII mRNA was not due to either mutations or hypermethylation of the promoter or gene [47, 48]. These findings indicate that inactivation of the TGFβ signaling pathway by the loss of TGFβRII gene expression may be common to SCLCs, and these tumors, like small intestinal NENs may be resistant to TGFβ1-mediated growth inhibition.

Therapeutic targeting of TGFβ is complicated by the variable anti-growth or pro-growth effects in different NENs. However, in cases where tumor growth is driven by TGFβ production, three approaches have been developed to inhibit TGFβ signaling; inhibitors of TGFβR, antisense oligonucleotides, or monoclonal antibodies to inhibit ligand-receptor interactions [34, 49]. Several agents (e.g., SD-208) have been developed that target receptor kinase activity and exhibit utility in limiting tumor invasion and metastasis in both pancreatic adenocarcinoma and melanoma nude mouse models [50, 51] (table 1). Since no clinical data is currently available it is unclear whether they could be effective in GEP-NENs. An alternative approach may be TGFβ-antisense which has shown promise both in preclinical (rat glioma models) [52, 53] and early clinical trials (high-grade gliomas) [54]. However, TGFβ-antisense agents have not been studied in NEN cell lines, and like TGFβ kinase inhibitors, their utility remains to be established.

In summary, the TGFβ superfamily is an important regulator of growth in NENs. Although it exhibits uniform inhibition of cell growth in the normal physiological setting, NEN

models show mechanisms of escape from TGFβ-mediated growth inhibition. Further, TGFβ appears to encourage growth in NENs, some of which exhibit high levels of TGFβ and TGFβR. To the best of our knowledge, therapeutic targeting of TGFβ has not been attempted in NENs.

Connective Tissue Growth Factor (CTGF): A Proliferative Regulator and Role in Fibrosis

CTGF, insulin growth factor binding protein (IGFBP) or CCN2, is a 38kDa, cysteine-rich secreted protein coded by chromosome 6q23.1 [55, 56]. This is one of the immediate-early response genes expressed after induction by growth factors or certain oncogenes [56]. CTGF has been identified in a variety of tumors of mesenchymal, epithelial, and lymphoid origin [56] and expression levels of transcripts and/or protein are positively correlated with bone metastasis in breast cancer [57], glioblastoma growth [58], poor prognosis in esophageal adenocarcinoma [59], aggressive behavior of pancreatic cancer cells [60], and invasive melanoma [61]. This gene is also over-expressed in a mouse transgenic model of gastric neuroendocrine cell carcinoma [62].

CTGF and Proliferation

CTGF signaling has been studied in NEN tumor models. In animal studies (*Mastomys* model), *CTGF* transcript and protein were over-expressed in gastric ECL tumor cells compared to normal ECL cells [63]; this growth factor stimulated tumor ECL cell proliferation but not normal cell proliferation and synergized the proliferative effects of EGF under *in vitro* conditions [63]. These effects were mediated via ERK1/2 phosphorylation and could be reversed by pharmacological inhibition of this pathway with PD98059 (fig. 3) [63]. These data suggested CTGF may play a role as a regulator of ECL cell proliferation. A follow-up *in vivo* study, where the CCK_2 receptor was inhibited during hypergastrinemia induced by the irreversible histamine 2 receptor antagonist, Loxtidine, identified a reduction of CTGF levels (and animals did not develop tumors) confirming that this growth factor played a role in tumor ECL cell proliferation [64]. A proliferative role for CTGF has been confirmed in the small intestinal NEN cell lines; KRJ-I responded with proliferation to CTGF (EC_{50} = 0.002 ng/ml), but no effect was noted on BON cell proliferation and little is known regarding the role of CTGF in the mechanistic regulation of BP-NENs proliferation [31].

CTGF signaling has also been studied in NENs. In gastric NENs, expression of CTGF mRNA and protein specifically differentiated Type 1 and 2 “gastrin-dependent” lesions from Type 3 “gastrin-independent” neoplasms [63], with over expression of CTGF in the more malignant Type 3 tumors [63] suggesting that CTGF may be related to autonomous (non-gastrin responsive) tumor growth

CTGF and fibrosis

In addition to functioning as a growth factor for NENs, CTGF has a fundamental role in mediating the fibrosis associated with small intestinal NENs. In particular, small intestinal NENs over-express CTGF mRNA and synthesize CTGF protein which was significantly elevated in tumors and blood of patients with clinically documentable fibrosis [65]. CTGF immunoreactivity was identified in >50% of tumor cells in 100% of lesions (n=42) with less expression in pancreatic NENs (14%) and BP-NENs (20%) [66]. Protein bands corresponding to full-length CTGF (36–42 kDa) were detected as was immunoreactive cells that expressed α-smooth muscle actin (α-SMA) in adjacent mucosa [66]. These results confirm a potential role for CTGF in myofibroblast-mediated fibrosis associated with these neoplasms, and indicate that CTGF may be a therapeutic target. Further, plasma CTGF is strongly related to valvular and mural carcinoid heart disease [67]; a significant inverse correlation was noted

between right ventricular function and plasma CTGF levels, patients with reduced cardiac function had higher plasma CTGF levels, and CTGF≥77 μg/L was identified as an independent predictor of reduced cardiac function (sensitivity and specificity of 88% and 69% respectively) [67]. In addition, plasma CTGF was elevated in patients with moderate to severe valvular regurgitation [67]. Both these studies identify that CTGF may play a role in neuroendocrine neoplasm-related mesenteric and cardiac fibrosis. The detection of elevated blood levels may provide a diagnostic opportunity to predict the development of fibrosis and pre-empt its local and systemic complications.

CTGF has been considered an attractive therapy for fibrosis-associated diseases. Neutralizing and single-chain variable fragment antibodies (scFv) against CTGF have efficacy in mouse models of fibrosis [68, 69]. Similarly, a phase I study in microalbuminuric diabetic kidney disease (DKD) (a progressive fibrotic disease) using a human monoclonal antibody against CTGF (FG-3019) identified that the urinary albumin/creatinine ratio (ACR) (a marker of efficacy) was significantly decreased [70]. However, no studies have been undertaken in NENs for fibrosis-associated diseases. The potential efficacy of this approach has been demonstrated in mouse xenograft studies with pancreatic cancer cells [71] where FG-3019 abolished CTGF-dependent tumor growth and inhibited lymph node metastases without any toxicity in normal tissue [71] (table 1). Alternatively, as this protein is a down-stream target of TGFβ1 [56], inhibiting the latter signaling pathway may have efficacy in reducing CTGF expression and diminishing its proliferative activity.

In summary, the presence of CTGF in tumors is associated with a malignant phenotype across a range of cancers. CTGF is present in many NENs and encourages growth in small intestinal NEN cell lines and a gastric NEN animal model. The presence of CTGF is associated with more malignant phenotypes in clinical gastric and Small intestinal NENs. As well as encouraging proliferation, CTGF is a pivotal mediator of NEN-associated fibrosis.

Conclusion

Proliferation of GEP- and BP-NENs is responsive to the growth factors EGF, TGFα, TGFβ and CTGF, and therefore maybe susceptible to therapeutics that target these receptors or associated signaling pathways e.g. BRAF/MAPK. Despite evidence from cell lines, animal models and clinical samples, there are no good examples of the use of receptor-tailored pharmacotherapies that target these growth factors in NENs. The multiple and over-lapping signaling pathways that characterize GEP-NENs (fig. 4), however, suggest targeting these tumors at a number of levels may be required to provide efficacy.

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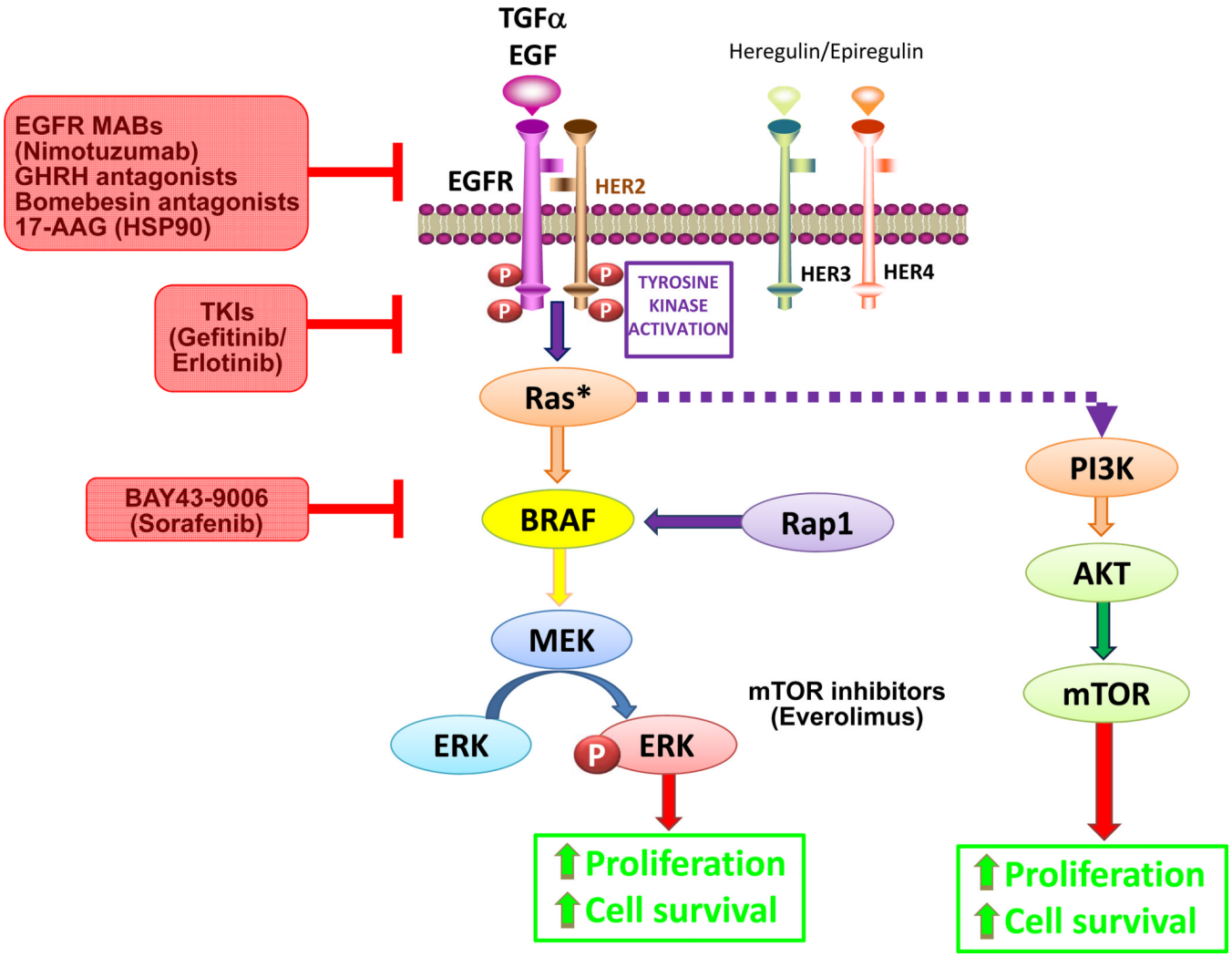

Fig. 1. Epidermal growth factor receptor and its growth regulation and inhibition

EGF and TGFα are typical growth factors that transduce growth signals via EGFR (HER1), which is one member of the ErbB family of receptor tyrosine kinases. Upon ligand binding, HER1 and HER2 dimerize, resulting in autophosphorylation and activation of the intracellular kinase domains which initiates the cascade of Ras, BRAF and MEKK. The latter phosphorylates MAPK (ERK) which activates transcription factors including ELK1 positively regulating proliferation and cell survival. This pathway is well studied in a variety of cancers and can be inhibited at a number of levels including the receptor (e.g. by Nimotuzumab or Cetuximab) the kinase itself (e.g. Gefitinib) or at the level of RAF (e.g. Sorafenib). The absence of susceptible mutations in EGFR might explain the lack of efficacy of Gefitinib in GEP- and BP-NENs. Mutations in TK are associated with cross-activation of the PI3K/AKT/mTOR pathway but this appears only to be a potential relationship and has not been demonstrated in NENs. Mutations in K-Ras occur in ~2% and may lead to loss of responsiveness to EGFR monoclonal approaches. The efficacy of other agents targeting different levels of this pathway in GEP- and BP-NENs is not currently known.

*=KRAS mutation

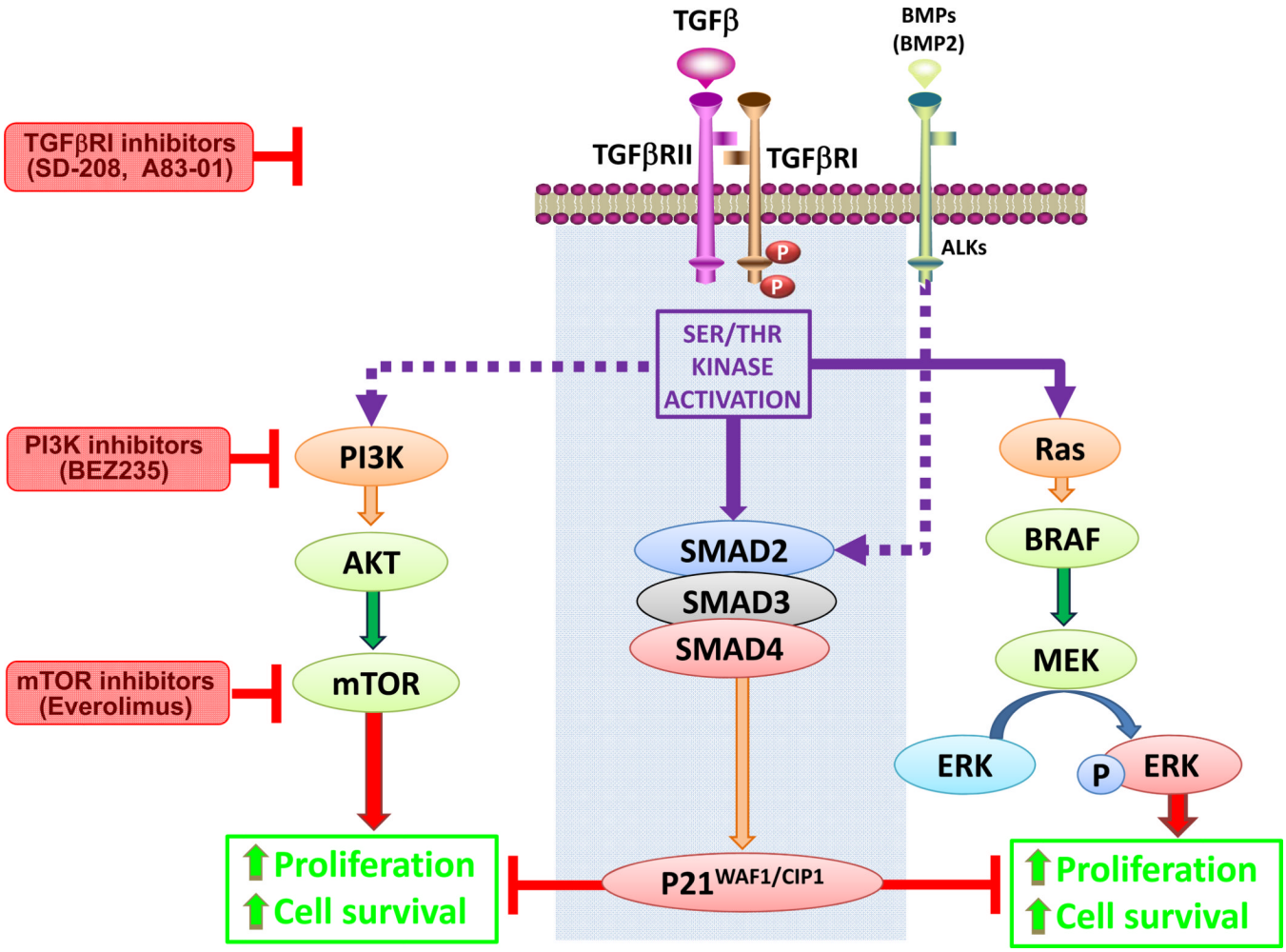

Fig. 2. Transforming growth factor beta receptor and its growth regulation and inhibition

Under physiological conditions, activation of TGFβR complex I and II following TGFβ binding results in G1 cell cycle arrest (through the inhibitory activity of $P21^{WAF1/CIP1}$) (*central panel*). These effects are mediated via the serine threonine kinase of the receptor complex, SMAD2,3,4 activation (through phosphorylation) and nuclear targeting of these transcription factors. BMP receptors are also associated with activation of the SMAD cascade. In NENs (and other tumors e.g., glioblastomas), nuclear targeting of SMAD is inhibited and cross-activation of growth-stimulatory pathways occur including the RAS/RAF/MAPK signaling pathway and the PI3K/AKT/mTOR pathways. Under these circumstances, TGFβ switches to a pro-proliferative role. No preclinical or clinical studies have been conducted using TGFβRI inhibitors in NENs, while other studies have demonstrated the utility of targeting the mTOR pathway. Solid lines: good evidence for pathway activation; dotted lines: incomplete evidence.

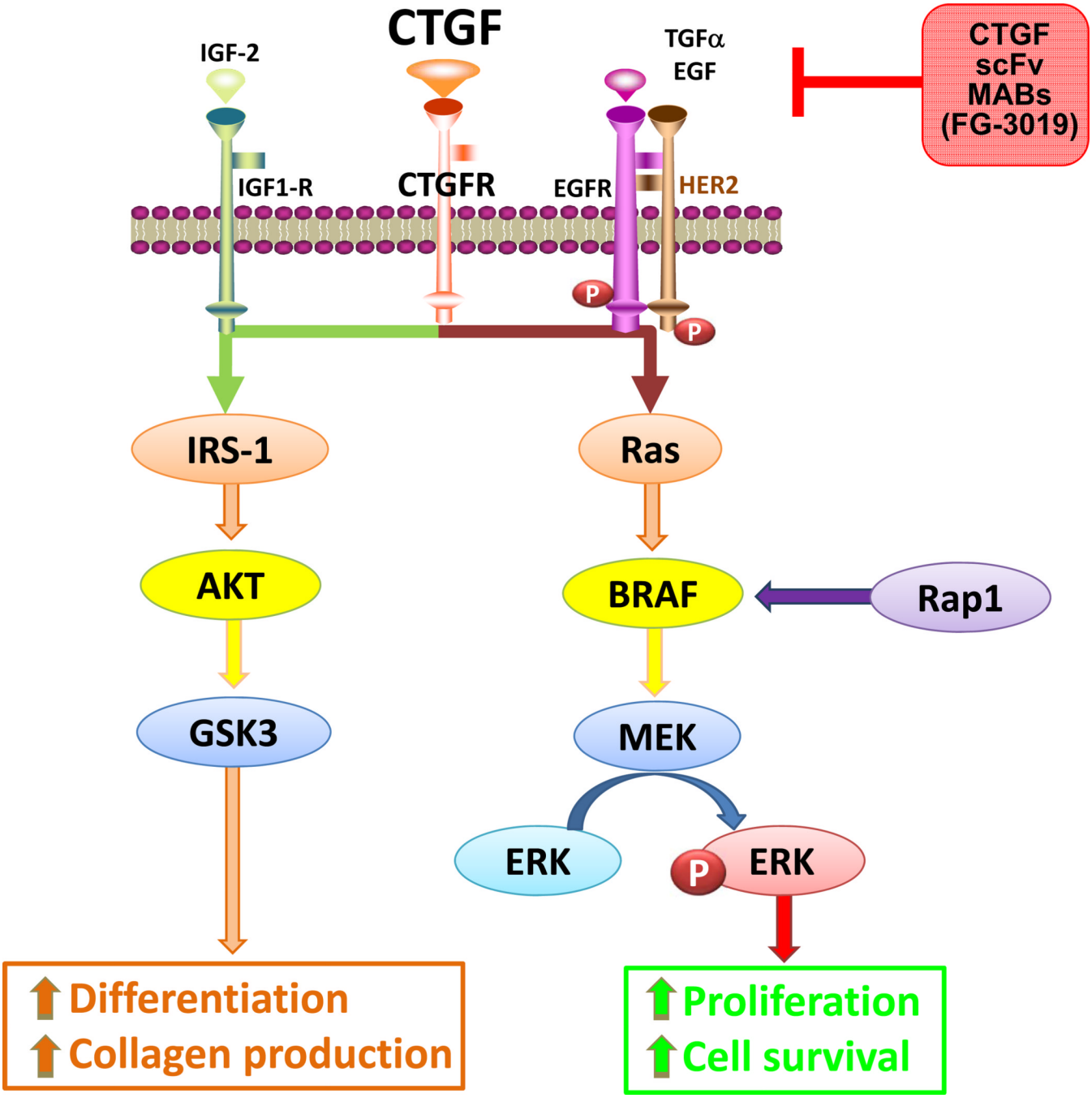

Fig. 3. Connective tissue growth factor regulations and fibrosis

CTGF binds its receptor CTGFR but may also bind IGF receptors. Activation of CTGFR augments both IGF-R and EGF-R signaling pathways. The former is associated with AKT/GSK3 activation and the production of collagen – a key event in fibrosis – the latter with growth-mediated cell proliferation. While monoclonal antibodies against CTGF have efficacy in preclinical and clinical studies of fibrotic iseases, targeting this receptor has not been undertaken in NENs.

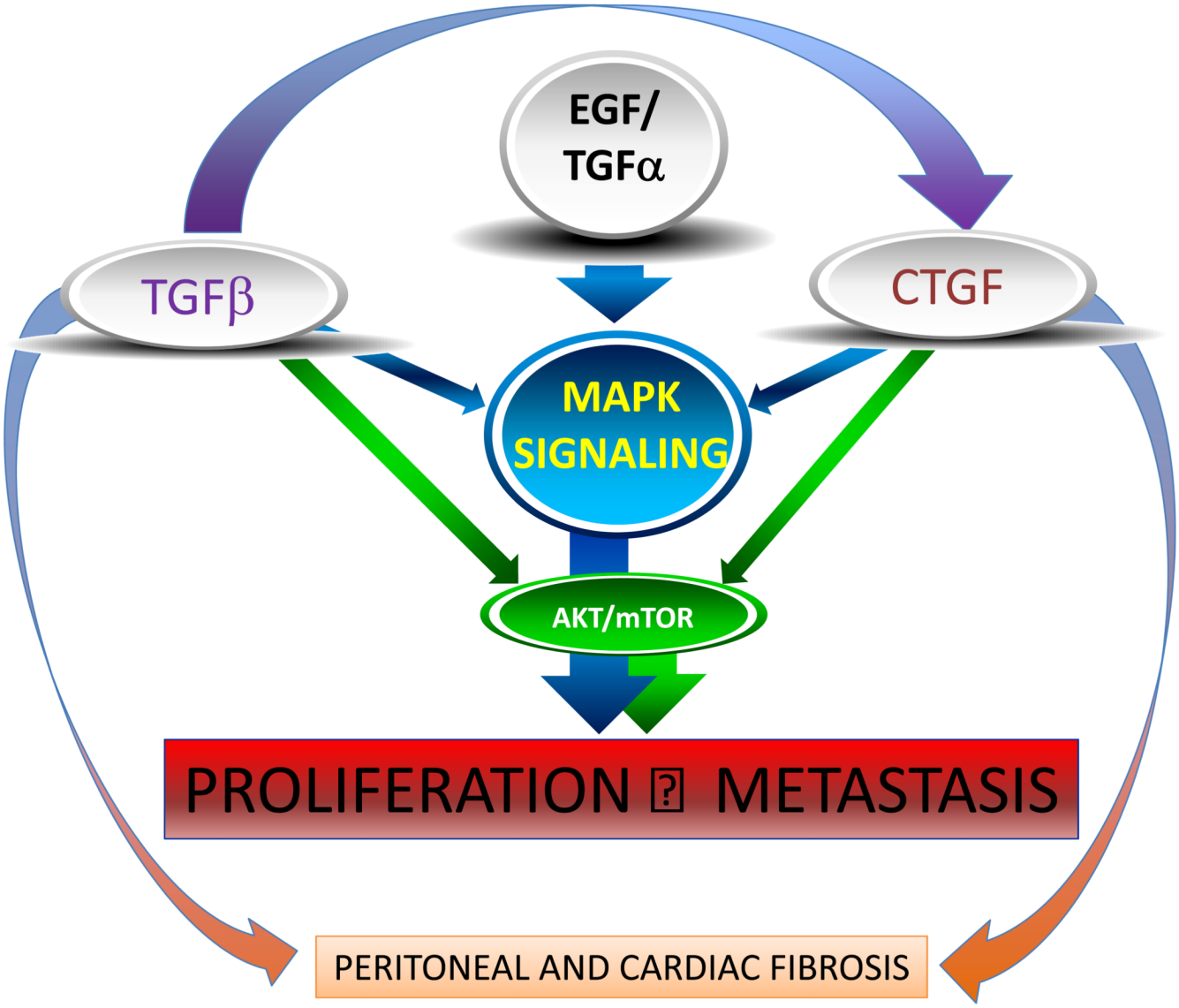

Fig. 4. Overview of common signal pathway activation in GEP-NENs

The signaling pathways activated either directly or due to cross-activation is MAPK and AKT/mTOR signaling. All ligands signal via these pathways to positively regulate proliferation and metastasis. Separately, an event that only occurs in a subset of GEP-NENs (Small-intestine NENs), TGFβ and CTGF activate fibrogenic pathways resulting in local (peritoneal) or distant (cardiac) disease. The common activation of RAS/RAF signaling suggests that therapeutic agents that target elements of this pathway may be potentially effective in NENs.

Table 1

Growth pathways as potential targets

	EGFR (HER1)-pathway	Therapy	TGFβR-pathway	Therapy	CTGF-R pathway	Therapy
Receptor	ErB family	Cetuximab	TGFβR II	SD-208 A83-01	CTGF-R	FG-3019
Ligand	EGF/TGFα		TGFβ1,- β2,- β3		CTGF	
Receptor activation	Tyrosine kinase	Gefitinib	Serine threonine kinase		LRP1 **	
Downstream	BRAF MEK ERK	Sorafenib	1.SMAD 2,3,4 2.PI3K AKT mTOR	BEZ235 Everolimus	Via EGF: BRAF/MEK/ ERK Via IGF1-R: IRS-1/AKT/ GSK3	Sorafenib
Function	-Proliferative gene expression -Cytoskeletal rearrangement -Increased cell proliferation		1.normal: G1 cell cycle arrest 2.cross activation: cell proliferation		GSK3 Via EGF: see EGF-R- Pathway Via IGF1-R: Differentiation Collagen production	

** Putative receptor is LRP1, but this is not confirmed.