Published in final edited form as:

Exp Cell Res. 2009 February 15; 315(4): 583–601. doi:10.1016/j.yexcr.2008.10.043.

ERBBs in the gastrointestinal tract: Recent progress and new perspectives

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Abstract

The gastrointestinal epithelium does much more than provide a physical barrier between the intestinal lumen and our internal milieu. It is actively engaged in absorption and secretion of salt and water via ion transporters, exchangers and selective ion channels. It is also a continuously self-renewing epithelium that undergoes ordered growth and differentiation along its vertical axis. From this dual perspective, we will consider the actions of the ERBB family of ligands and receptors in the maintenance of gastrointestinal homeostasis and discuss instances when the actions of this family go awry such as in cancer and Ménétrier's disease.

Keywords

ERBBs; Colorectal cancer; Ménétrier's disease

Introduction

This review is intended to highlight recent important advances in our understanding of roles for ERBBs in gastrointestinal biology and pathology. We published a similar review in *Gastroenterology* in 1995 [1], and it is gratifying to note the progress that has been made in this field since that time. More recently, we provided an update on EGF receptor (EGFR) ligands in this journal [2]. Due to space constraints, this review perforce is highly selective, and we apologize in advance to our colleagues for omissions of relevant published work. The focus will be on selected topics where substantial progress has been made, e.g., transactivation of the EGFR, interactions between microbes in the gut and EGFR and insights into gastrointestinal homeostasis based on relevant genetic manipulations in the mouse. We also will point out gaps in our knowledge and emerging areas of opportunity. This review is not intended to be comprehensive. For example, the liver and pancreas are not discussed, although the interested reader is referred to several reviews related to the pancreas [3,4].

General background

The four members of the ERBB family are depicted in Fig. 1. In contrast to the other family members, ErbB3 lacks intrinsic tyrosine kinase activity but contains 6 PI3-K binding sites within its cytoplasmic tail. Family members can homo- and heterodimerize. Preformed dimers may exist [5], but ligand binding results in oligomerization of the receptors, activation of intrinsic tyrosine kinase activity and transphosphorylation of tyrosine residues on the partnered receptor that initiates a complex signal transduction cascade. ErbB2 lacks a ligand but is thought to be the preferred binding partner of the other ErbBs [6,7]. All seven of the vertebrate EGFR ligands are synthesized as type-1 transmembrane proteins (Fig. 1). Upon insertion into the plasma membrane, they undergo ectodomain cleavage by cell surface proteases to release soluble ligand that then engages the receptor. In some cases, membrane-bound ligand is not cleaved, and can signal through adjacent receptor-bearing cells (juxtacrine signaling), a process best documented for HB-EGF [8–10]. Considerably more detail on these topics is presented elsewhere in this monograph.

In the normal adult human gut and in a battery of polarizing human colorectal cancer (CRC) cell lines, EGFR is found predominantly at the basolateral surface [11], as presumably are ErbB2–4 [12]. The pathophysiological significance of apical EGFRs, if any, is less certain [13]. At sites of acute and chronic injury, basolateral EGFRs become exposed to the luminal environment and may be accessed by endogenous luminal EGFR ligands such as EGF or the administration of EGF enemas [14]. Playford and Wright introduced the apt term “luminal surveillance peptide” to describe the action of EGF that is secreted into the gut lumen by the salivary gland and Brunner's glands [15]. A similar phenomenon was described by Welsh et al. for apically released heregulin accessing basolateral ErbB3 and 4 in the damaged lung [16]. All components of the EGFR axis (defined as the proximal events in activation of EGFR) are found at the basolateral compartment of two human polarizing CRC cell lines (HCA-7 and Caco-2) derived from well-differentiated CRCs [17]. That is, TGF α and amphiregulin are delivered selectively to the basolateral surface where they are cleaved by TNF- α converting enzyme (TACE)/a disintegrin and metalloprotease (ADAM)-17 to release mature soluble ligand that then binds basolaterally restricted EGFRs.

TGF α is touted to be the most abundant EGFR ligand in the GI tract [18]. David Lee et al. assessed mRNA expression of all of the EGFR ligands (except epigen) from the stomach to the colon in the mouse [19]. Less is known about the precise localization of the ErbB family and its ligands along the vertical axis of the gut. Our impression is that in the normal colon there is a decreasing gradient of expression for TGF α and EGFR from the luminal surface to the crypt base with a sharper gradient for TGF α than EGFR. However, following fasting and refeeding in the rat, TGF α immunoreactivity extends towards the crypt base in the colon (RJ Coffey, unpublished observation). Bertagnoli et al. have observed a similar gradient of EGFR immunoreactivity from the villous tip to the crypt base in the mouse small intestine [20].

A systematic analysis at the protein level of the ERBB family and its cognate ligands along the horizontal and vertical gradient of the gastrointestinal tract has not been performed. Such an analysis should assess total and phosphorylated ERBBs; ideally, this would delineate phosphorylation status of specific tyrosine residues. This may be particularly relevant in the neoplastic state since we have observed that Ras transformation of RIE-1 cells *in vitro* results in downregulated, yet tyrosine hyperphosphorylated, EGFR [21]. Such a systematic analysis of receptors and ligands has, thus far, been limited due to technical difficulties. Western blotting requires adequate amounts of tissue, and immunohistochemistry is fraught with issues related to specificity, quantification, preservation of antigenicity and fixation artifacts. Application of advances in mass spectrometry may be a useful direction to pursue in this area, especially in

the assessment of the phosphorylation state of specific tyrosine residues in the cytoplasmic tails of the different ERBBs.

Transactivation of the EGFR

Transactivation of the EGFR was defined operationally by Ullrich et al. as G Protein-Coupled Receptor (GPCR)-triggered signaling (specifically MAP kinase and SHC phosphorylation) that was blocked by EGFR tyrosine kinase inhibition. As described below, it initially was thought to be ligand-independent; however, it subsequently was shown to be due to metalloproteinase-regulated cell surface cleavage and release of mature soluble EGFR ligand. Ligand engagement of EGFR activated its intrinsic tyrosine kinase and initiated downstream signaling. Instances of GPCR-triggered, EGFR ligand-independent EGFR activation have been reported, but their mechanistic underpinnings are less clear. Furthermore, one can envision GPCR-mediated EGFR signaling that may not depend upon EGFR tyrosine kinase activation. For example, GPCR activation of Src tyrosine kinase activity may lead to direct phosphorylation of tyrosine (Y)845 within the EGFR cytoplasmic tail [22]. In this case, EGFR may act as a docking site for SH2-containing proteins and subsequent signal transduction. To further complicate matters, active Src may also activate TACE/ADAM17 and lead to ligand-dependent EGFR activation [23]. Finally, it should be recalled that there can be GPCR-independent, ligand-dependent transactivation of the EGFR. One example is the phorbol ester TPA, presumably acting as a general cell surface sheddase [24]. However, this action of TPA may be counter-balanced by a PKC-mediated phosphorylation of threonine (T)654 within the cytoplasmic tail of the EGFR that results in receptor internalization and downregulation.

What follows is a brief historical review of GPCR transactivation of the EGFR. In 1996, Ullrich et al. first reported that the GPCR agonists endothelin-1 (ET-1), lysophosphatidic acid (LPA) and thrombin induced tyrosine phosphorylation of EGFR (and ErbB2/c-neu) within 2 min of their administration to Rat-1 fibroblasts [25]. Given the rapid kinetics of this effect, they concluded that EGFR activation must be ligand-independent; however, they were not able to formally exclude this possibility because monoclonal antibodies (mAbs) to block ligand binding to rat EGFR were not available at that time. They postulated that the effect was due to an intracellular release of a negative restraint (likely a phosphatase) on receptor tyrosine kinase activity.

In collaboration with Steve Wiley, we published in early 1999 that a broad spectrum, hydroxamate-based, metalloproteinase inhibitor, batimastat, could reduce the growth of HCA-7 cells and, importantly, that addition of EGF reversed the growth inhibitory effect of batimastat [26]. Similar results were observed in a human mammary epithelial cell line (HMEC); moreover, in these cells, the metalloproteinase inhibitor reduced the levels of TGF α and AR in the medium, and a combination of batimastat and an EGFR mAb that blocked ligand binding resulted in cooperative growth inhibition of these cells. This work identified a metalloproteinase-regulated EGFR activity that was mediated by endogenous EGFR ligands.

Later in 1999, Ulrich et al. revisited their initial contention that GPCR transactivation of the EGFR was ligand-independent [27]. Using Rat-1 cells expressing a chimeric human EGFR ectodomain fused to PDGFR transmembrane and cytoplasmic tail, they demonstrated that EGFR transactivation was prevented by a mAb that blocked ligand binding to human EGFR. They implicated HB-EGF as the EGFR ligand mediating this transactivation by exploiting the observation that HB-EGF acts as a receptor for diphtheria toxin. CRM197 (a non-toxic mutant of diphtheria toxin that specifically inhibits the mitogenic effects of cells producing HB-EGF) was able to block EGFR transactivation by GPCR agonists in COS-7 and HEK 293 cells; however, CRM197 did not affect exogenous EGF-induced EGFR tyrosine phosphorylation. Likewise, batimastat was able to abrogate GPCR agonist and TPA transactivation of the EGFR

in COS-7 cells. Finally, batimastat was shown to prevent bombesin (another GPCR agonist) and TPA-induced tyrosine phosphorylation of EGFR in a prostate cancer cell line, PC3. Consistent with our earlier work [26], batimastat was able to reduce baseline tyrosine phosphorylation of EGFR in these cells.

These studies established that GPCR transactivation of EGFR was rapid and EGFR ligand-dependent but did not identify the specific metalloproteinase. By noting that TPA-, but not GPCR-induced tyrosine phosphorylation of EGFR, was sensitive to protein kinase C inhibition, they suggested that at least two distinct metalloproteinase-dependent transactivation pathways existed. As noted above, work from our lab has shown that TACE/ADAM-17 localizes to the basolateral surface of polarized HCA-7 cells and that a selective TACE/ADAM-17 inhibitor WAY022 blocks cell surface release of AR and TGF α in these cells [17].

These original observations have spawned a small cottage industry related to GPCR transactivation of EGFR in general [28] and in the gastrointestinal tract in particular (see Table 1). The role of EGFR transactivation in chloride secretion will be discussed in more detail as it illustrates several points we wish to highlight (Fig. 2). Barrett et al. identified one of the first examples of GPCR transactivation of EGFR in the gut. Using a polarizing human colorectal cancer cell line (T84), they showed that the muscarinic (M) agonist carbachol (CCh), a stable analogue of the neurotransmitter acetylcholine, transactivates EGFR in a TGF α -dependent manner [29]. By binding to the basolateral M3 receptor (a GPCR), CCh stimulates apical chloride secretion by a mechanism that requires increases in intracellular calcium (left panel in Fig. 2A). The active secretion of chloride ions provides the driving force for intestinal fluid secretion. Binding of CCh to the M3 receptor also results in a rapid metalloproteinase-dependent release of TGF α into the basolateral medium and avid consumption by basolateral EGFRs (right panel in Fig. 2A), activation of EGFR and transmission of an ERK-mediated signal that blunts apical chloride secretion. The CCh-mediated transactivation of EGFR and subsequent suppression of chloride secretion does not appear to involve PI3-K, unless PTP1B, which dephosphorylates a number of EGFR tyrosine residues, is suppressed [30].

Interestingly, administration of EGF and TGF α to the basolateral compartment also blunted chloride secretion but through ERK and PI3-K signaling and, in the latter instance, this may involve protein tyrosine phosphatases, including PTP1B [30]. The authors suggested that exogenous EGF and TGF α may alter the activity of phosphatases that then dephosphorylate certain key EGFR residues differently than CCh-regulated, TGF α -mediated EGFR transactivation. In these experiments, exogenous EGF was more potent than TGF α and elicited EGFR–ErbB2 heterodimerization that was not seen with equivalent concentrations of TGF α . This work demonstrates a growth-independent effect of endogenous TGF α and supports our original contention that TGF α acts locally [31]. In contrast, activation of PAR-2 results in increased cAMP and ligand-independent transactivation of EGFR that stimulates CFTR-mediated chloride secretion (Fig. 2B) [32]. In normal mouse colon, EGF inhibits cAMP-dependent ion transport [33], but, in the background of inflammation, exogenous EGF also stimulates sodium absorption (Fig. 2C) [34]. These studies emphasize the potential of EGFR phosphorylation and dephosphorylation mechanisms to regulate recruitment of alternate downstream signaling pathways following EGFR activation by different stimuli.

Based on these observations, it is tempting to speculate on potential mechanism(s) underlying the diarrhea that is seen in 25% of patients receiving the EGFR neutralizing mAb cetuximab. One could envision that EGFR blockade impairs the dampening of CCh-induced chloride secretion but also abrogates the sodium absorption that takes place in the setting of ongoing inflammation, a state that is increasingly linked to neoplasia. Obviously, one must also consider the effects of EGFR blockade on growth and repair processes that may also contribute to the

diarrhea associated with pharmacological blockade of the EGFR, as well as its effects on microbes in the gut (see next section).

A question that arises is how many other signaling pathways act through EGFR. Recent studies by Steve Wiley et al. in human mammary epithelial cells (HMEC) have found that EGFR is a central coordinator of many disparate signaling inputs. In addition to activating GPCRs (LPA receptors, calcium-sensing receptors, purinergic receptors), administration of VEGF, GH, TNF α and IGF results in EGFR activation [35]. Moreover, ERK phosphorylation in response to general stressors (sorbitol and anisomycin) was dependent on EGFR kinase activity. However, not all roads converge upon EGFR since ERK phosphorylation following HGF activation of Met was not dependent on EGFR signaling. On the other hand, in two colon cancer cell lines (LoVo and Caco-2), Tarnawski et al. reported that PGE₂-induced c-Met phosphorylation was dependent upon EGFR activation [36]. These differing results may reflect the contextual nature of signaling responses.

We wish to make several final points. An emerging theme is the convergence of signaling pathways at the level of EGFR, and that the EGFR axis acts as a central hub in integrating many, but certainly not all, cell surface signaling events. One interesting example is the feedforward action of EGFR activation and its upregulation of COX-2. Amphiregulin upregulates COX-2 expression. The COX-2 product, PGE₂, induces expression of amphiregulin that binds and activates EGFR that, in turn, further upregulates COX-2 [37]. It is important to note that GPCRs can signal independently of EGFR [38]. GPCR transactivation of EGFR has been documented *in vivo* [39]; however, it is only 10% as potent as exogenous EGF in activating EGFR. Deconstructing the contribution of EGFR activation to GPCR signaling will be complicated by identifying whether the effect is ligand-dependent or -independent and, if it is ligand-dependent, which ligand and which metalloproteinase cleaves that ligand. Such an analysis may be further complicated by the phenomenon of auto- and cross-induction [40,41], as well as the recent identification of a tyrosine kinase-independent action of EGFR [42]. Finally, one must also consider how GPCR transactivation of EGFR is terminated. Recent studies have identified PKC α -mediated feedback inhibition as one such mechanism [43–45].

Gut microbes and EGFR

Although EGFR and other ErbB receptors are involved in normal gastrointestinal development and homeostasis (discussed below), they are also utilized by various infectious agents in causing human disease. EGFR is employed by bacteria and viruses as a means of facilitating invasion or cell entry, promoting cell survival early in the infectious process and also may play a role in neoplastic progression that is associated with certain infections, such as *Helicobacter (H.) pylori*. This section will review some of the ways in which microorganisms interact with the ERBB system within the gut.

Enteropathogenic *Escherichia coli* (EPEC) is a diarrhea-causing pathogen that is responsible for significant morbidity and mortality worldwide. Evidence suggests that EGFR signaling may play a role in *E. coli* disease pathogenesis [46–48]. Such a link was suggested initially when it was observed that oral administration of EGF to New Zealand white rabbits prevented *E. coli* colonization of the small and large intestine and prevented diarrhea and weight loss in the rabbits [47]. This observation may also have relevance to cetuximab-associated diarrhea. Rather than being due to altered Cch-mediated chloride secretion, EGFR blockade may facilitate pathogen-induced diarrhea. Additionally, internalization of EPEC into human renal epithelial cells was inhibited by administration of the EGFR tyrosine kinase inhibitor AG1478 [48].

In C2_BBE intestinal epithelial cells infected with EPEC, there was a progressive increase in phosphorylation of EGFR Y1068. Furthermore, C2_BBE monolayers treated with filter-sterilized supernatants of wild-type EPEC also stimulated Y1068 phosphorylation (although it was comparatively less than when EPEC was present), suggesting that a secreted element may be acting to induce this phosphorylation event. EGFR tyrosine phosphorylation was inhibited by administration of AG1478, and this also attenuated EPEC-induced Akt and ERK1/2 phosphorylation. When EGFR was inhibited by AG1478 in the setting of EPEC infection, cell survival was decreased and IL-8 production was inhibited. These findings suggest that EPEC can utilize the EGFR to promote host cell survival and increase IL-8 production [46]. It is possible that apoptosis of host cells early in bacterial infection is detrimental to the pathogen, and that promoting host cell survival is important in disease pathogenesis. *Pseudomonas aeruginosa* has also been shown to activate EGFR and downstream ERK and PI3-kinase activity in a similar manner [49].

Clostridium difficile (*C. diff*) acts through a similar pathway. *C. diff* secretes two major toxins, toxin A and toxin B, which are both high molecular weight exotoxins and are responsible for the watery diarrhea characteristic of *C. diff* infection. Toxin B induces EGFR-mediated activation of ERK1/2 phosphorylation, leading to release of IL-8 from human colon cell lines, NCM460 and HT-29. Inhibition with AG1478 or EGFR neutralizing mAb blocks EGFR and ERK activation, along with release of IL-8. TGF α was implicated as the ligand responsible for activating EGFR in this system [50].

EGFR appears to be important in the invasion of another bacterial pathogen, *Salmonella typhimurium*. *In vitro* exposure of *S. typhimurium* to two human intestinal epithelial cell lines, Henle-407 and T84, induces EGFR tyrosine phosphorylation [33,51]. A strain of *S. typhimurium* which is non-invasive due to a mutation in the *invA* gene does not induce this phosphorylation. Addition of EGF to cultured Henle-407 cells allowed internalization of the invasion-defective strain of *S. typhimurium* with mutant *invA*. However, addition of EGF did not cause internalization of adherent, but non-invasive, *Escherichia coli* [51]. These data indicate that *S. typhimurium* can induce EGFR tyrosine phosphorylation which may contribute to its internalization and invasive phenotype. In contrast, McNeil et al. studied *S. typhimurium in vitro* and *in vivo* using *Egfr* (*waved-2* [*wa2*]) mice that have a spontaneous mutation in the EGFR tyrosine kinase domain resulting in markedly reduced (approximately 90%), but not absent, intrinsic EGFR tyrosine kinase activity. *S. typhimurium* invaded fibroblasts from *wa2* mice as efficiently as fibroblasts from control animals. Moreover, *in vivo*, *S. typhimurium* both invaded the gastrointestinal mucosa and penetrated through to the spleen in *wa2* mice [52]. Taken together, these data indicate that *S. typhimurium* has the ability to interact with EGFR and induce phosphorylation, but this does not appear to be a necessary step in invasion. Alternatively, the modest residual EGFR tyrosine kinase activity in *wa2* mice may be sufficient to mediate this effect.

Similarly, there is conflicting data on the importance of EGFR on the entry of human cytomegalovirus (CMV) into epithelial cells. In 2003, Wang et al. proposed that EGFR was a receptor responsible for cell entry of the virus [53]. CMV can activate both MAP-kinase [54, 55] and PI3-kinase [56], but a receptor for cell entry and signaling was not identified at the time. Wang confirmed that CMV interacted with EGFR and activated downstream targets of EGFR signaling such as PI3-kinase and Akt. This effect was inhibited by administration of AG1478. Transfecting EGFR-negative MDA-MB453 cells with EGFR cDNA rendered non-susceptible cells susceptible to CMV cell entry. CMV interacted with EGFR through glycoprotein B, its principal surface envelope protein, and glycoprotein B promoted the hetero-oligomerization of EGFR with ErbB3 [53]. On the other hand, Isaacson et al. found no correlation between EGFR expression and CMV cell entry in a variety of cell lines (NHDF and HEL fibroblasts, ARPE-19 retinal epithelial cells, UtMVEC and HUVEC endothelial cells)

nor did EGFR neutralizing mAb block CMV entry into these cell lines [57]. Again, this may indicate that CMV has the capacity in some cases to utilize the EGFR for cell entry and signaling, but that is not necessarily an obligatory step.

In contrast to the above pathogens which cause acute illness, *H. pylori* is an organism that colonizes the stomach during childhood and can lead to lifelong infection in the host if untreated [58]. Approximately half of the world's population is infected with *H. pylori*, and it is known to be associated with diseases such as atrophic gastritis, peptic ulcer disease [59–61], lymphoma of mucosa-associated lymphoid tissue (MALT) and gastric adenocarcinoma [62, 63]. Among the responses of gastric epithelial cells to infection with *H. pylori* is increased expression of EGFR [64], increased expression of EGFR ligands amphiregulin and HB-EGF [65,66] and activation of EGFR signaling [67] which leads to upregulation of COX-2 and VEGF in gastric epithelial cells [68].

Busiello et al. studied the virulence factors associated with changes in EGF-related peptides and EGFR in two human gastric cancer cell lines, MKN28 and AGS [69]. They found that the soluble protein gamma-glutamyltranspeptidase (GGT), a virulence and colonizing factor for *H. pylori*, retained the ability to upregulate HB-EGF mRNA and protein expression after fractionation from medium of cultured *H. pylori* strains. Acivicin, a selective GGT inhibitor, counteracted the upregulation of HB-EGF by *H. pylori*, and upregulation of HB-EGF was not observed following infection of gastric epithelial cells with an isogenic mutant GGT-deficient strain of *H. pylori*. This effect appeared to be mediated through PI3-kinase and p38 kinase, as their blockade with LY294002 or SB203580, respectively, inhibited *H. pylori*-induced upregulation of HB-EGF, whereas blockade of MAP kinase with PD98059 had no such effect [69].

In contrast to the above observations showing increased EGFR activity related to *H. pylori* infection, Schiemann et al. observed that infection with *H. pylori* was associated with reduced mRNA expression of TGF α , amphiregulin and HB-EGF [70]. Chronic gastritis due to *H. pylori* was associated with decreased antral EGFR binding compared to healthy controls, as assessed by [¹²⁵I]-EGF binding. Moreover, in 19 patients with chronic *H. pylori* infection, levels of these ligands increased after eradication therapy. The authors hypothesized that reduced EGFR-mediated gastroprotection may contribute to the pathogenicity of *H. pylori* infection [70].

These findings highlight the controversy surrounding the impact of *H. pylori* on EGF-related peptide expression in human gastric mucosa. Other groups have found that HB-EGF and amphiregulin are upregulated in the gastric mucosa of individuals infected with *H. pylori* [71,72], and that this overexpression correlates with an increase in the mitotic index of the gastric mucosa, as assessed by PCNA immunostaining [72]. However, Persico et al. noted decreased expression of TGF α in the gastroduodenal mucosa of individuals infected with *H. pylori*, similar to the observation of Schiemann et al. [73]. In light of these findings, it is clear that more research is needed to further elucidate the interaction between *H. pylori*, EGF-related peptides and EGFR signaling.

The cytotoxin-associated gene A product (CagA) is another *H. pylori* virulence factor that is associated with higher grades of gastric inflammation and an increased risk of gastric cancer [74]. Increased expression of EGFR is induced by *H. pylori* infection at both the mRNA and protein level. This effect is limited to CagA+ strains of *H. pylori* and those with an intact type IV secretion system [64]. Upregulation of HB-EGF is also more strongly induced by CagA+ strains of *H. pylori* than by CagA– strains [67]. It is unknown whether these findings contribute to the increased oncogenic potential of CagA+ strains of *H. pylori*.

Finally, it is useful to recall that HB-EGF serves as the receptor for the B fragment of Diphtheria toxin [75].

Homeostasis

In this section, we will review the gastrointestinal consequences of genetic engineering of the ERBB family and its ligands in the gut. The cumulative evidence suggests that EGFR signaling contributes to mucosal integrity in response to damage and other provocative maneuvers. Targeted disruption of the *Egfr* in mice results in strain-dependent effects. Using different inbred strains, Threadgill et al. observed implantation lethality in CF-1 mice, midgestation lethality in 129/Sv mice and perinatal lethality in CD-1 mice with these mice living past weaning, at which time the overall gut architecture appeared normal [76]. However, Miettinen et al. found that *Egfr* null mice on a mixed background exhibited features of necrotizing enterocolitis (NEC) [77]. Moreover, a body of literature supports that EGFR ligands may prevent NEC [78]. However, these ameliorative effects of EGFR ligands do not prove that loss of *Egfr* in mouse gut directly causes NEC. It may be that the NEC phenotype was secondary to multi-organ failure and the overall debilitated state of these mice, or, alternatively, to impaired barrier function and repair capability leading to enteropathogen susceptibility as described above. Experiments comparing *Egfr* mutant mice in conditions with known bacterial compositions will be required to elucidate whether NEC is a direct or indirect consequence of *Egfr* deficiency. Nonetheless, more recent genetic data using conditional ablation of *Egfr* in the gut epithelium using an *Egfr* conditional allele with the Villin-Cre transgene suggests that NEC and other homeostatic defects are secondary to loss of *Egfr* which creates a sensitized background [79].

Hypomorphic, homozygous *wa2* mice have increased susceptibility to DSS-induced colitis [80], as do TGF α null and epiregulin null mice [81,82]. Conversely, TGF α transgenic mice exhibit decreased injury in response to DSS [83]. The lack of susceptibility to DSS-induced colitis in the EGF, TGF α and AR triple null mice is likely due to differences in strain backgrounds or health status of the respective colonies [19]. These triple null mice, however, do exhibit spontaneous erosions in the duodenum.

Wa2 mice have reduced intestinal adaptation following 50% small bowel resection [84,85]. Mice overexpressing an assembled mouse EGF cDNA downstream of a rat intestinal fatty acid binding promoter have 2-fold increased levels of EGF protein in the gut and exhibit enhanced intestinal adaptation following small bowel resection [86]. Intestinal adaptation is not affected in TGF α null mice [87], a finding that likely reflects ligand redundancy under these experimental conditions.

The function of ErbB2 and ErbB4 in gastrointestinal homeostasis has not been evaluated because embryos deficient for either die prenatally due to developmental defects of the heart [88,89]. However, a conditional allele for ErbB3, which also leads to prenatal lethality when deficient, has allowed its role to be evaluated. Mice lacking ErbB3 in the intestinal epithelium, created by combining an ErbB3 conditional allele with the Villin-Cre transgene, develop normally but show a high frequency of bifurcated villi [90]. The developmental cause of this morphological change is not known but the mutant mice appear to have normal absorption based upon body weight. Mice deficient for ErbB3 in the intestinal epithelium show elevated susceptibility to DSS-induced colitis.

Esophagus

Increased EGFR signaling appears to play a role in the pathogenesis of both squamous cell carcinoma (SCC) and adenocarcinoma of the esophagus. Increased EGFR expression is common in SCC of the esophagus and has been associated with poor outcomes [91,92]. A

retrospective review of 107 cases of squamous cell carcinoma of the esophagus demonstrated that 68.2% of cancers had overexpression of EGFR by immunohistochemistry [91]. Increased expression was associated with a higher incidence of vascular invasion, higher local recurrence rates and lower overall survival. Increased EGFR expression has also been associated with higher rates of local spread to regional lymph nodes [92]. Although the majority of SCCs show overexpression of EGFR by immunohistochemistry, only 21% of primary tumors exhibit DNA amplification of EGFR by Southern blot analysis [93].

Rustgi et al. evaluated the effects of EGFR overexpression by retroviral transduction in normal human epithelial cell lines EPC1 and EPC2. Compared with normal and GFP-transduced primary human epithelial esophageal cells, cell cultures overexpressing EGFR demonstrated a thicker epithelium and basal cell hyperplasia, as well as increased cell migration. These effects were markedly diminished by addition of the EGFR tyrosine kinase inhibitor AG1478 to the medium of these organotypic cultures. The effect of EGFR on cell migration appeared to be dependent upon EGFR-induced upregulation of matrix metalloproteinase-1 (MMP-1) [94], an effect which was mediated by the JAK-STAT pathway [95]. *In vivo*, a transgenic mouse model of EGFR overexpression in the oral and esophageal squamous epithelia was also studied. Compared with age-matched littermates, transgenic mice overexpressing EGFR in the esophageal mucosa demonstrated increased proliferation in the basal and suprabasal cells, consistent with the findings noted in cell culture [94].

More recently, the same group has used human epithelial cells as a platform to recapitulate esophageal squamous cell cancer. This was accomplished by retroviral-mediated transduction of EGFR, the catalytic subunit of human telomerase (*hTERT*) and *p53*^{R175H} into normal human esophageal epithelial cells. These transduced cells demonstrated increased migration and invasion, and, in an organotypic 3-dimensional culture system, they formed a high grade dysplastic epithelium with malignant cells invading the stromal extracellular matrix. The invasive phenotype was mediated, in part, by matrix metalloproteinase-9 (MMP-9), and invasion was attenuated by knockdown of both AKT1 and AKT2 [96].

EGFR also appears to play a role in Barrett's-associated adenocarcinoma of the esophagus. Wang et al. showed that EGFR was expressed in 32% of adenocarcinomas of the esophagus, and that expression of EGFR was associated with higher T stage, the presence of lymph node metastases and also with a trend toward shorter disease-free survival in multivariate analysis [97]. In addition to increased EGFR immunoreactivity, mutations in EGFR have been detected in 11.7% of esophageal adenocarcinomas and 14.2% of cases of Barrett's esophagus [98]. In this study, mutations consisted of the missense L858R mutation and in-frame deletion delE746–A750. Both of these mutations are located in the tyrosine kinase domain of the receptor and previously have been characterized as activating EGFR mutations in non-small cell lung cancer which predict responsiveness to EGFR tyrosine kinase inhibitors [99–102]. The fact that these mutations are present in Barrett's esophagus, as well as adenocarcinoma, suggests that there is a subset of adenocarcinomas of the esophagus in which mutation of EGFR may play an early role in tumorigenesis [98]. TGF α and EGF appear to be involved in mucosal defense in chronic reflux esophagitis [103,104], which is known to be associated with development of esophageal adenocarcinoma [105]. This provides a plausible mechanism through which chronic reflux could promote the neoplastic process in part by stimulating EGFR signaling.

The role of ErbB2, ErbB3 and ErbB4 are less well defined in the pathogenesis of esophageal carcinoma. The ErbB2 gene is amplified in approximately 21–32% of esophageal adenocarcinomas arising out of Barrett's esophagus and 5–27% of Barrett's specimens with high grade dysplasia [106,107]. Overexpression of ErbB2 by FISH was observed in 19% of esophageal adenocarcinomas and correlated with poor prognosis [108]. No Barrett's samples

with low grade dysplasia or intestinal metaplasia without dysplasia demonstrated increased copy number of ErbB2 by quantitative RT-PCR [107]. This indicates that overexpression of ErbB2 is involved in a subset of esophageal adenocarcinomas, although it is a relatively late step in the metaplasia–dysplasia–carcinoma sequence. One uncontrolled Phase I/II study evaluated the use of trastuzumab, a humanized monoclonal antibody that acts on the ErbB2 receptor, in patients with locally advanced esophageal adenocarcinomas that overexpressed ErbB2 by immunohistochemistry. Nineteen patients were enrolled, and median survival was 24 months with a 2-year survival of 50% [109], numbers that are comparable with historical controls. Due to the small proportion of adenocarcinomas that overexpress ErbB2, accrual for this study was slow, and it was felt that an adequately powered Phase III study would be difficult to perform.

ErbB3 has also been shown to be upregulated in esophageal adenocarcinoma through a proteomic approach using high-resolution, two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization, time-of-flight and tandem mass spectrometry analysis (MALDI TOF MS) [110]. This finding was then validated using qRT-PCR and immunohistochemical analysis on a tissue array. Fifty-one percent of tumors had increased ErbB3 mRNA expression, and 42% had increased immunohistochemical staining for ErbB3 [110]. mRNA expression for ErbB4 and heregulin has been detected in esophageal mucosa [111], but there is no clear evidence that alterations in their expression play a role in esophageal carcinogenesis.

Stomach

In the stomach, enhanced EGFR signaling has been implicated in the pathogenesis of Ménétrier's disease (hypoproteinemic hypertrophic gastropathy). In adults, this is a rare, acquired, progressive overgrowth state of the stomach characterized by enlarged gastric folds in the body and fundus of the stomach, hypochlorhydria and protein loss across the gastric mucosa. Characteristic histological findings include foveolar hyperplasia with an expansion of surface mucous cells at the expense of acid-producing parietal cells and pepsinogen-producing chief cells [112]. The condition appears to be associated with an increased risk of malignancy, although the magnitude of this risk is not known [113–118].

Evidence from mice and humans supports a role for excess EGFR signaling in the pathogenesis of Ménétrier's disease [119,120]. TGF α suppresses gastric acidity, stimulates gastric epithelial cell proliferation and induces gastric mucus production, all of which are characteristic features of Ménétrier's disease. Transgenic mice that overexpress TGF α exhibit many features of Ménétrier's disease including enlarged gastric folds, foveolar hyperplasia and reduced numbers of chief cells and parietal cells with hypochlorhydria [121]. Patients diagnosed with Ménétrier's disease exhibit increased immunoreactivity for TGF α [122]. To date, a systematic analysis of other EGFR ligands has not been performed in this disease nor has there been a report of targeted overexpression of any of the other EGFR ligands in the mouse stomach.

Perhaps the strongest evidence for the role of EGFR signaling in the pathogenesis of Ménétrier's disease comes from pharmacologic blockade of EGFR in the treatment of patients with this disease. We now have experience in treating eight patients with Ménétrier's disease with cetuximab, a monoclonal antibody that inhibits the activity of the EGFR. All of the patients treated thus far as part of an FDA-approved clinical trial with four weekly doses of cetuximab have demonstrated clinical improvement in their predominant symptom. They also have exhibited biochemical evidence of significant improvement in a number of parameters that include decreased gastric wall thickness by CT scan, reduced proliferation in the involved stomach (as determined by reduced active phosphorylated MAP kinase) and increased parietal cell mass with decreased gastric pH (WH Fiske and RJ Coffey, unpublished observation). As

assessed by electron microscopy, the integrity of tight junctions in the gastric mucosa of a patient with Ménétrier's disease was improved after treatment with cetuximab [123].

In adolescents, there is an abrupt onset, spontaneously reversible form of Ménétrier's disease that has been linked epidemiologically to CMV infection (see Gut Microbes and EGFR section). We recently encountered a patient with acute onset, clinically and histologically documented Ménétrier's disease whose signs and symptoms resolved in the two-month interval from diagnosis to evaluation for entry into the cetuximab trial. We identified CMV nuclear inclusions in his gastric epithelial cells and acute and convalescent serum titers for CMV confirmed the diagnosis (WH Fiske and RJ Coffey, unpublished observation). Thus, CMV-induced Ménétrier's disease can occur in immuno-competent adults. We now exclude patients from our Ménétrier's disease trial if they have evidence of active *H. pylori* or CMV infection.

ErbB family members also play a role in gastric carcinogenesis. Some of these effects are mediated through infection with *H. pylori* and are discussed in the section on Gut microbes and EGFR. However, there is also evidence that ErbB family members play a more direct role in gastric carcinogenesis as well.

Immunoreactive EGF is present in approximately 26% of gastric cancers, and the presence of EGF in gastric cancer is associated with a greater degree of gastric wall invasion and lymph node metastasis; the 5-year survival is worse for patients with EGF-positive tumors [124]. EGFR has also been identified in a small subset of gastric cancers, although this is uncommon. In one study, high levels of EGFR immunoreactivity (2+ or 3+ by immunohistochemical staining) were present in 9 of 413 tumor samples (2.2%), and low level expression (1+ staining) was present in 8.2% of samples tested [125]. In tumors that demonstrated high levels of expression by immunohistochemistry, FISH almost always demonstrated evidence of gene amplification as the mechanism for overexpression [125]. Inhibition of EGFR signaling does appear to be effective in reducing the growth of human gastric cancer cells both *in vitro* and *in vivo* [126]. However, data evaluating the clinical efficacy of pharmacological blockade of EGFR are lacking. There has been one uncontrolled Phase II clinical trial evaluating cetuximab in combination with FOLFIRI as a first-line treatment of advanced gastric or gastroesophageal junction (GEJ) adenocarcinoma [127]. This regimen appeared to be active in gastric cancer with an acceptable rate of toxicity. Further randomized clinical trials evaluating cetuximab in gastric cancer will need to be completed in order to assess its effectiveness.

ErbB2 has been shown to be overexpressed in human gastric carcinomas approximately 8–14% of the time [128–130] and is associated with a worse prognosis [130–133]. Overexpression of ErbB2 by immunohistochemistry is associated with greater depth of invasion, intestinal-type carcinoma (not diffuse or mixed/anaplastic growth pattern), the presence of liver metastases and more advanced stage [128,133]. Trastuzumab, a monoclonal antibody against ErbB2, has been studied in both *in vitro* and *in vivo* models of gastric cancer. Trastuzumab inhibited the growth of ErbB2-overexpressing SNU-216 cells. Phosphorylation of ErbB2 and its downstream targets STAT3, AKT and ERK were also inhibited. This effect was synergistic with cisplatin, and an additive effect was seen with trastuzumab plus 5-FU or trastuzumab plus oxaliplatin [134]. In nude mouse xenografts, trastuzumab significantly suppressed the growth of NCI-N87 cells [135]. The combination of trastuzumab with other chemotherapeutic agents appears to be more effective than anti-cancer agents alone in xenograft models [136]. There are anecdotal reports of the efficacy of trastuzumab in individuals with gastric carcinoma [137,138], although large scale clinical trials are lacking.

ErbB3 may play a role in the progression to more dedifferentiated gastric cancers. NUGC-4 cells, derived from a signet ring carcinoma of the stomach, have a constitutively phosphorylated ErbB3 that binds to phosphatidylinositol 3-kinase (PI3-kinase), although similarly derived

HSC60 cells do not demonstrate phosphorylated ErbB3 or an association of ErbB3 with PI3-kinase [139]. In contrast, phosphorylated ErbB3 is not detected in more differentiated cancer cell lines such as SCH, MKN28, MKN74 and HSC57, although total ErbB3 can be detected.

ErbB4 may play a role in gastric carcinogenesis, although its role is not clear. In one study, ErbB4 was not found to be expressed at the mRNA level in normal gastric mucosa. However, it was expressed in 4 of 6 gastric cancers evaluated [111]. This same study performed immunohistochemical analysis as well. In the normal stomach, immunostaining for ErbB4 was detected in surface epithelial cells but not gastric fundic or pyloric glandular cells. ErbB4 staining was identified in all gastric cancers evaluated and appeared to be most intense in signet ring cancers [111]. Mutations of ErbB4 in gastric cancer are rare. In a panel of 180 human gastric cancers, mutations were found in only 1.7% of cases [140].

Intestine and colorectum

We have employed a genetic approach to identify a role for EGFR signaling in a post-initiation, early stage of intestinal neoplasia. *Apc^{Min}* (*multiple intestinal neoplasia*) mice are heterozygous for an ENU-induced mutation in the *Apc* gene and develop multiple polyps in the small intestine, and occasionally colon, at 3 months of age. *Egfr* null mice exhibit embryonic and/or perinatal lethality on most genetic backgrounds, but *wa2* mice harbor a spontaneous mutation in the tyrosine kinase domain that produces a hypomorphic allele of *Egfr*. As mentioned above, *wa2* mice exhibit a 90% reduction in EGFR tyrosine kinase activity but are viable and fertile. We crossed *wa2* mutant mice to *Apc^{Min}* mice and evaluated tumor multiplicity at 1 and 3 months of age [141]. We were able to demonstrate that EGFR does not have a role in tumor initiation (at 1 month there were just as many initiated events as determined by the number of microadenomas and crypts containing cells with nuclear β -catenin). There was, however, a 90% reduction in the number of macroadenomas at 3 months of age. Thus, EGFR signaling plays a critical role in the advancement from micro to macroadenomas in what we refer to as the establishment phase of intestinal tumorigenesis. The few polyps that do form on a *wa2* background are equivalently-sized to *Apc^{Min}* polyps arising in *Egfr* wild-type mice and expand in an EGFR-independent manner over a 9 month follow-up as determined by size and by histology in a small cohort of mice by linear regression analysis. Fichera et al. also have observed that EGFR signaling is required for early colonic tumor formation in mice treated with the carcinogen azoxymethane [142]. The precise mechanism by which EGFR signaling contributes to tumor establishment is not known but is believed to be through reduced proliferation based upon results of Fichera et al. Treatment of established human colonic tumors in nude mouse xenografts (HCA-7 and HCT-116) with a small molecule irreversible inhibitor of EGFR signaling, EKB-569, resulted in concentration-dependent tumor growth inhibition, revealing a role for EGFR signaling during later stages of tumorigenesis.

In addition to a Ménétrier's disease-like phenotype, MT-TGF α mice develop hyperplasia in the small intestine and colon. Bill Dove et al. recently observed increased tumor multiplicity in the jejunum of MT-TGF α transgenic mice crossed to *Apc^{Min}* mice. There was no increase in the size or net growth of established tumors, supporting a role for TGF α , like EGFR, in the establishment phase of intestinal neoplasia. Interestingly, MT-TGF α mice exhibited 4-fold less apoptosis in the jejunum than non-transgenic mice. However, the effects of the TGF α transgene on tumor multiplicity in *Apc^{Min}* mice were region-specific in that no effects on tumor number were observed in the remainder of the small intestine or colon. We are not aware of other transgenic mice overexpressing EGFR ligands that have been crossed to *Apc^{Min}* mice. Conversely, epiregulin null mice are the only EGFR ligand-deficient mice that have been crossed to *Apc^{Min}* mice; homozygous epiregulin null mice showed a dose-dependent trend toward reduced tumor multiplicity, but it was not statistically significant [82].

There is ample preclinical evidence for EGFR contributing to various aspects of colorectal neoplasia. Fidler et al. have proposed that the EGFR in tumor-associated endothelial cells may be the target of the EGFR tyrosine kinase inhibitor [143,144]. EGFR amplification and mutations in the EGFR tyrosine kinase domain occur rarely in colorectal cancer [197]. The frequency of the VIII EGFR variant in colorectal cancer is uncertain; two groups report widely variant results [145,146]. These differences may be methodologic in nature; using a newly developed, more sensitive, one-step PCR reaction, Dr. Albert Wong has observed the VIII transcript in 9/16 colorectal tumors [147] (Albert Wong, personal communication).

The EGFR-targeting immunoglobulin IgG1 monoclonal antibody, cetuximab, and the IgG2 monoclonal antibody, panitumumab, are both approved in patients with metastatic CRC. Cetuximab has been shown to be active as a single agent as well as in combination with irinotecan in patients with chemorefractory metastatic CRC [148]. The activity of cetuximab in combination with irinotecan- and oxaliplatin-based therapy in the first-line setting has been demonstrated in phase II studies and a phase III study [149,150]. Panitumumab has been shown to be active as single agent in chemorefractory metastatic CRC [151].

However, since only approximately 10% of patients respond to monotherapy, intense efforts have been devoted to identification of biomarkers that predict responsiveness or non-responsiveness to these EGFR-neutralizing monoclonal antibodies. Levels of total and phosphorylated EGFR have not proven to be reliable predictors of responsiveness, findings that may reflect the imprecision of the tools available. Conversely, retrospective [152–155] and prospective [156] studies have identified mutant KRAS in CRC as a powerful predictor of non-responsiveness to EGFR mAbs. Wild-type KRAS in CRC appears to be required for responsiveness, although only a small proportion of patients will respond even when this is present [153,157]. High levels of amphiregulin and epiregulin mRNA expression in the tumor also predict responsiveness to cetuximab [156]. It appears that patients with mutant KRAS tumors do not exhibit upregulation of amphiregulin or epiregulin; however, only a subset of wild-type KRAS tumors exhibit upregulation of these EGFR ligands [158]. High amphiregulin and epiregulin expression with wild-type KRAS primary CRCs correlates with response and survival benefit after treatment with cetuximab and irinotecan for metastatic disease [158]. Mechanism(s) underlying these observations are unclear. Amongst other possibilities, the sensitivity of these EGFR ligand-overexpressing tumors may reflect “growth factor addiction” by the tumor. It is unclear whether cetuximab resistance is conferred by mutant KRAS itself or by the company it keeps. If due directly to mutant KRAS, it may reflect that RAS is downstream of EGFR, despite preclinical evidence that mutant RAS upregulates EGFR ligands [159].

Using a battery of human colorectal cancer cell lines, Mariadason et al. recently reported that simultaneous activation of RAS and PIK3CA pathways confer maximal resistance to cetuximab. These investigators found that some CRC cell lines with mutant KRAS did respond to cetuximab, a result that is at odds with the preponderance of the clinical data. Other preclinical studies suggest that strategies that target Src [160] and HIF-1 α [161] may work in concert with cetuximab; in the latter case, siRNA knockdown of HIF-1 α was able to restore sensitivity to cetuximab in mutant RAS-transfected A431 cells.

In contrast to cetuximab, the reversible EGFR tyrosine kinase inhibitor gefitinib has little, if any, activity in patients with advanced colorectal cancer [162]. Nevertheless, Baselga et al. have reported a response to combined irressa and cetuximab in 9/14 individuals with advanced CRC (J. Baselga, personal communication). In addition, *in vitro*, combined pharmacological blockade of TACE/ADAM-17, ligand uptake and EGFR tyrosine kinase activity resulted in cooperative growth inhibition in HCA-7 cells [17].

The role of ErbB2 (HER-2/*neu*) in CRC is less clear than for EGFR. In 1997, Kapitanovi et al. performed an immunohistochemical analysis of normal colon, hyperplastic polyps, tubular adenomas, tubulovillous adenomas and adenocarcinomas and observed that these tissues differed in their expression of ErbB2 [163]. Only 14% of normal colons exhibited moderate or strong immunostaining for ErbB2 in contrast to 31% of hyperplastic polyps, 65% of tubular adenomas, 100% of tubulovillous adenomas and 84% of adenocarcinomas. Furthermore, the intensity of staining among adenocarcinomas correlated with Dukes' stage at diagnosis, metastasis-free survival and post-operative survival. Among tumors initially classified as Dukes' stage A or B which also demonstrated moderate or strong staining for ErbB2, 76% developed distant metastases during follow-up, and these patients had significantly shorter survival than those with weak staining for ErbB2. In another study, tumors from patients with lymph node metastasis were more likely to exhibit ErbB2 immunostaining than those tumors with no lymph node involvement [164].

Trastuzumab, a monoclonal antibody with activity against ErbB2, has been studied in both *in vitro* and *in vivo* models of CRC [165]. Growth of heregulin-1-stimulated HCA-7 cells was inhibited by both celecoxib and trastuzumab. However, the combination appeared to be more effective than either drug given alone. In Matrigel, trastuzumab alone had little effect on colony number or size, whereas colony size was significantly reduced by the combination of trastuzumab and celecoxib compared to celecoxib alone. Trastuzumab also inhibited the growth of HCA-7 xenografts in nude mice. Again, this effect was more pronounced when used in combination with celecoxib.

However, this high rate of ErbB2 expression has not been observed in other studies. In one Phase II clinical trial, only 8% of patients screened for HER2/*neu* by immunohistochemistry had overexpression demonstrated by moderate or strong staining. Partial responses to treatment with trastuzumab were seen in 5 of 7 evaluable patients, but this study was stopped early due to slow accrual [166]. As a result, there are no large randomized clinical trials evaluating the effectiveness of trastuzumab in the treatment of patients with ErbB2-overexpressing CRCs.

Unlike in the case of breast cancer, the utility of using HER-2 overexpression to predict response to HER-2 inhibition in colorectal cancer has been called into question. It has been observed that the ability of the HER-2 monoclonal antibody 4D5 to inhibit cancer cell proliferation and survival *in vitro*, or tumorigenicity *in vivo*, varies by cell line. For example, 4D5 inhibits proliferation in Caco-2 and HCA-7 but not DLD-1 or HT29 cells. Importantly, the relative steady-state expression of HER-2 by immunohistochemistry failed to predict the susceptibility of cancer cell lines to HER-2 inhibition. This may indicate that other methods are needed to predict which patients may be likely to benefit from HER-2 inhibition [167].

Recently, Threadgill et al. have reported that targeted homozygous deletion of *ErbB3* in the mouse intestine using villin-Cre mice in combination with floxed *ErbB3* results in a more dramatic reduction in tumor burden than homozygous *wa2* mice crossed to *Apc^{Min}* mice [90]. Although there was a 90% reduction in tumor multiplicity in both strains, the intestinal tumors were much smaller in *ErbB3 Apc^{Min}* mice, and no colonic tumors developed. There was reduced PI3-K/AKT signaling in the tumors with increased caspase3-mediated apoptosis. Loss of *ErbB3* in the intestinal epithelium has a much more pronounced antitumor effect than loss of EGFR suggesting that non-EGFR combinations contribute to the majority of tumors (Fig. 3).

The role of ErbB4 (HER4) in CRC has not been as well studied as other receptors. In a retrospective immunohistochemical review of 106 colorectal cancers, ErbB4 was expressed at the membrane in 18.9% and in the cytoplasm in 28.3%. Tumors staining positive for ErbB4 were associated with lymph node involvement, although overall outcomes did not differ

[168]. Mutations in ErbB4 can occur in CRC but are rare, happening in approximately 2.9% of cases. All of those detected in one study by Soung et al. were missense mutations [140]. Interestingly, a germline polymorphism in the 5' promoter region of ErbB4 (-782 G>T) has been associated with increased risk of CRC (as well as breast cancer). In this study, the G allele showed higher protein binding affinity than the T allele in competition experiments, and the T allele had a more than 2.5-fold decrease in *in vitro* promoter activity as assessed by reporter gene assays using MDA134 and MDA157 cells for transfection. Taken together, these findings suggest that differences in these observed polymorphisms have implications on ErbB4 promoter activity and may lead to the observed association with CRC [169].

Areas of opportunity

Ligand delivery/unpeeling the onion

An understudied area is the cell surface trafficking of EGFR ligands in polarized epithelial cells. This is likely to be physiologically relevant based on the recent observation that isolated recessive renal hypomagnesemia is due to a mutation in the cytoplasmic tail of pro-EGF that disrupts basolateral sorting of pro-EGF [170]. This leads to insufficient stimulation of basolateral EGFRs in the proximal tubule of the kidney, resulting in impaired activation of the Mg²⁺ channel TRPM6 (transient receptor potential cation channel, subfamily M, member 6) and magnesium wasting in the kidney. We have studied trafficking of EGF, TGF α and amphiregulin in polarized MDCK cells and TGF α and amphiregulin in polarizing colorectal cancer cell lines (reviewed in reference [2]).

The studies with TGF α are the most advanced. We have found that Naked2 acts as a *cargo* recognition and *targeting* (CaRT) protein for basolateral delivery of TGF α [171,172]. Naked2 is a member of the Naked family (*Naked Cuticle* in *Drosophila* and vertebrate Naked1 and -2) whose members act as antagonists of canonical Wnt signaling. Naked2 recognizes basolateral sorting motifs in the cytoplasmic tail of Golgi-processed TGF α , coats TGF α -containing exocytic vesicles and directs these vesicles to the basolateral corner of polarized epithelial cells where the vesicles dock and fuse in a myristoylation-dependent manner (Fig. 4A). In myristoylation-deficient G2ANaked2-expressing MDCK cells, these vesicles are directed to the basolateral surface but are unable to fuse at the basolateral surface, leaving these vesicles (and TGF α) trapped in the cytoplasm. Amphiregulin, however, is delivered properly to the basolateral surface in these Naked2 mutant cells, suggesting that it uses a different basolateral sorting machinery. We have exploited the trapped nature of these vesicles to biochemically enrich and flow sort G2ANaked2-EGFP vesicles from MDCK cells. By LC/LC-MS/MS, we have identified 389 proteins associated with this subset of basolaterally targeted vesicles [173], thus setting the stage for rapid advances in this field.

Moreover, we have found that TGF α upregulates Naked2 in an EGFR-independent manner [174]. Naked2 is a short-lived protein with a half-life of 1 h due to constitutive ubiquitin-mediated proteasomal degradation by AO7, a ring finger E3 ligase (Fig. 4B). However, upon interacting with the cytoplasmic tail of TGF α , Naked2 is protected from degradation. Thus, TGF α ensures its proper delivery to the basolateral surface of polarized epithelial cells by stabilizing its CaRT Naked2. Recent work indicates that expression of Naked2, in contrast to Naked1 [175], is markedly reduced in over 60% of CRCs compared to adjacent normal colon (RJ Coffey and W Ding unpublished observation). However, Naked2 levels are maintained in adenomas, a finding that indicates loss of Naked2 is a late event in CRC.

Distinct biological roles among the EGFR ligands

Ebner and Derynck first reported that EGF and TGF α exhibit clear differences in disposition upon ligand-receptor internalization [176]. Others have reported that the individual EGFR

ligand determines the specificity of downstream signaling [27,177,178]. We have shown that AR, but not TGF α , causes an EMT-like phenotype when added to MDCK cells [179,180]. Amphiregulin null mice, but not TGF α null mice, develop gastric cancer [181]. The judicious use of overexpression and knockdown strategies of ERBB receptors and cognate ligands *in vitro* and *in vivo* should help to elucidate distinct roles for the different ligands.

Stromal epithelial interactions

It will be informative to examine the relative contribution among ERBBs and their ligands in stromal epithelial crosstalk in the gastrointestinal tract during development and in maintenance of homeostasis in the adult [182]. It is instructive that stromal EGFR is critical for mammary gland development [183].

Conclusion

The true physiological role of this family of receptors and their cognate ligands will come from understanding their actions *in vivo*. Reductionist approaches *in vitro* will continue to be useful, but attempts to use 3D models and reconstitution experiments combining different tissue components (epithelial cells, pericryptal fibroblasts and other tissue elements) will be used increasingly to better simulate *in vivo* conditions.

Acknowledgments

The authors thank Declan McCole, Marco Romano, Scott Kuwada, Brad Warner and Albert Wong for helpful discussions and Wei Ding for assistance in the preparation of figures. This work was supported by R01CA092479, U01CA105417 and P50CA106991 to DT and R01CA46413, GI Special Program of Research Excellence (P50CA95103) and Mouse Models of Human Cancers Consortium (U01CA084239) to RJC. RJC also acknowledges the generous support of the Peter Powell Fund.

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Fig. 1.

Depiction of ERBBs and their cognate ligands. See text for details. AR, amphiregulin; BTC, betacellulin; EPR, epiregulin.

Fig. 2.

Simplified schema depicting effect of EGFR signaling on overall chloride secretion. Except for CFTR, it is not intended to represent specific chloride or sodium transporters. Panels A and B summarize studies performed in polarized T84 cells. (A) Left panel, carbachol (CCh) binds and activates basolateral GPCR M3 muscarinic receptors that stimulate rapid, yet transient, calcium-dependent apical chloride secretion. CCh activation of M3 receptors also triggers ectodomain cleavage of proTGF α by TACE. The soluble ligand is avidly captured by basolateral EGFRs; this transmits an incompletely understood signal that dampens chloride secretion. Right panel, upon CCh stimulation, there is TACE-mediated rapid ectodomain cleavage of TGF α and the soluble ligand is avidly captured by basolateral EGFRs (adapted

from Fig. 7 in reference [29]). (B) In contrast to calcium-mediated chloride secretion, increased intracellular cAMP (achieved e.g. by PAR-2, VIP, dibutyl cAMP, forskolin) stimulates slower onset, more sustained chloride secretion by a PKA-mediated direct effect on the CFTR chloride channel. Depicted here is activation of basolateral PAR-2 that results in Src-dependent transactivation of the EGFR. In this instance, there appears to be ligand-independent EGFR activation that contributes to chloride secretion. (C) In normal mouse colon, addition of EGF inhibits cAMP-dependent ion transport; however, in the setting of inflammation, EGF stimulates sodium reabsorption. In the inflamed mouse colon, this effect is blunted by tetrodotoxin, indicating that the effect is mediated, at least in part, through neurons. Ⓟ indicates activated, tyrosine phosphorylated EGFR.

Fig. 3.

Receptor combinations contributing to CRC growth. Results from inhibiting EGFR or conditional deletion of ErbB3 is consistent with a model whereby EGFR-containing dimers contribute to proliferation, while ErbB3 dimers largely contribute to survival. CRC growth requires both signals. When EGFR is blocked, tumors have reduced proliferation. However, tumors that lack EGFR have normal growth rates, presumably using other ErbB signals. When ErbB3 is lost, both a proliferation and survival signal are lost, resulting in a dramatic reduction in tumorigenesis.

Fig. 4. Model for TGF α -induced Naked2 stabilization. (A) Naked2 acts as a CaRT to escort TGF α -containing vesicles to the basolateral corner of polarized epithelial cells where they dock and fuse in Naked2 myristoylation-dependent manner. (B) In the absence of TGF α , Naked2 is ubiquitinated by AO7 and undergoes rapid proteasomal degradation. When TGF α binds Naked2, there is decreased binding of AO7 to Naked2 and Naked2 is protected from degradation. This action of TGF α is EGFR-independent. Figure is adapted from reference [172].

Table 1

Transactivation of EGFR in the GI Tract

Cell type	Stimulus	Receptor	EGFR ligand	Metallo-proteinase	Effect	Ref
T84	CCh	M3R	TGF α	TACE	↓ Chloride secretion	[29]
T84, SCBN	SLIGRL-NH2	PAR-2	Independent	Independent	↑ Chloride secretion	[32]
HT29-CL.16E	SLIGKV-NH2	PAR-2	ND	ND	↑ Mucus secretion	[184]
HT-29	Thrombin	PAR-1	TGF α	ND	↑ Proliferation	[185]
HCA-7, HCT-8	Bile acids (CA, DCA)	ND	AR	TACE	↑ PGE $_2$	[17]
NCM460	Substance P	NK-1R	TGF α	TACE	↑ Proliferation	[186]
Caco-2	MIP-3 α	CCR6	AR	TACE	↑ Proliferation	[187]
Caco-2	IL-8	IL-8RA/B	HB-EGF	ND	↑ Migration proliferation	[188]
KATO III	IL-8	IL-8RA/B	HB-EGF, AR	ADAM 10	↑ ϕ EGFR	[189]
AGS	Histamine	H2R	ND	ND	↑ MMP-1	[190]
LoVo	LPA	LPA2R	ND	ND	↑ COX-2	[191]
HT-29, LIM1215	ET-1	ET $_A$ R	ND	ND	↑ Proliferation	[192]
IEC-18	ANG-II	AT $_1$ R	ND	ND	↑ Proliferation	[193]
RGM1, Caco-2	PGE $_2$	EP receptor	TGF α	ND	↑ Proliferation	[194]
HCT-116	PGE $_2$	EP2R	Independent	Independent	↑ Survival	[195]
LS-174T	PGE $_2$	EP receptor	Independent	Independent	↑ Migration	[196]

Abbreviations: PAR, Protease-activated receptor; ND, Not determined; CA, Cholic acid; DCA, Deoxycholic acid; NK, Neurokinin; MIP, Macrophage-inflammatory protein; LPA, Lysophosphatidic acid; ET, Endothelin; ANG, Angiotensin; AT, Angiotensin; EP, Prostaglandin E.