ERBB4 is over-expressed in human colon cancer and enhances cellular transformation

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Abstract

The ERBB4 receptor tyrosine kinase promotes colonocyte survival. Herein, we tested whether ERBB4’s antiapoptotic signaling promotes transformation and colorectal tumorigenesis. ERBB4 alterations in a The Cancer Genome Atlas colorectal cancer (CRC) data set stratified survival, and in a combined Moffitt Cancer Center and Vanderbilt Medical Center CRC expression data set, ERBB4 message levels were increased at all tumor stages. Similarly, western blot and immunohistochemistry on additional CRC tissue banks showed elevated ERBB4 protein in tumors. ERBB4 was highly expressed in aggressive, dedifferentiated CRC cell lines, and its knockdown in LIM2405 cells reduced anchorage-independent colony formation. In nude mouse xenograft studies, ERBB4 alone was insufficient to induce tumor establishment of non-transformed mouse colonocytes, but its over-expression in cells harboring Apc⁰𝑚𝑚 and v-Ha-Ras caused a doubling of tumor size. ERBB4-expressing xenografts displayed increased activation of survival pathways, including epidermal growth factor receptor and Akt phosphorylation and COX-2 expression, and decreased apoptotic signals. Finally, ERBB4 deletion from mouse intestinal epithelium impaired stem cell replication and in vitro enteroid establishment. In summary, we report that ERBB4 is over-expressed in human CRC, and in experimental systems enhances the survival and growth of cells driven by Ras and/or WNT signaling. Chronic ERBB4 over-expression in the context of, for example, inflammation may contribute to colorectal carcinogenesis. Tumors with high receptor levels are likely to have enhanced cell survival signaling through epidermal growth factor receptor, PI3K and COX-2. These results suggest ERBB4 as a novel therapeutic target in a subset of CRC.

Introduction

The ERBB4 type I receptor tyrosine kinase is widely expressed across mammalian epithelial tissues, including the gastrointestinal tract (¹). Like other ERBBs [epidermal growth factor receptor (EGFR)/ERBB1, HER2/ERBB2 and HER3/ERBB3], it is activated by growth factor ligands of the heregulin- and EGF-related families, resulting in receptor homo- or hetero-dimerization and phosphorylation on cytoplasmic tyrosine residues (²). Depending on cell type and experimental conditions, ERBB4 activation can promote a broad range of cellular responses, including proliferation, differentiation, apoptosis, survival and migration (³–⁶). In the normal mammalian intestinal and colonic epithelium, data on ERBB4 are limited but suggest a selective role in cell survival rather than mitogenesis. Furthermore, the selective ERBB4 ligand neuregulin-4 inhibits tumor necrosis factor (TNF)-induced colonocyte apoptosis both in vitro and in vivo, without affecting cell proliferation or migration (⁷).

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Although it shares significant homology with EGFR/ERBB1, ERBB2 and ERBB3, ERBB4 also has a number of features that distinguish it from other tyrosine kinases. It recognizes a broader set of ligands than other ERBBs (8), yet it binds only a selective suite of downstream cytoplasmic signaling partners (9). Furthermore, proteolytic cleavage of the receptor can result in generation of an 80 kDa intracellular domain (ICD) fragment that translocates to the nucleus and participates in transcriptional regulation (10). Thus, in diseases where its expression or activity is altered, ERBB4 represents a unique molecular target for therapeutic manipulation with potential for greater selectivity/specificity than other receptor tyrosine kinases.

Similar to other growth factor receptors, ERBB4 expression is altered in many cancers. However, in contrast with ERBB1-3, the role of ERBB4 in tumorigenesis remains incompletely understood and may to some extent be tissue dependent (3,11,12). In the gastrointestinal tract, the data currently available suggest an oncogenic role (13,14). However, a mechanistic link between ERBB4 and colon epithelial cell transformation has not been demonstrated.

In this study, we addressed the fundamental question of whether the observed changes in ERBB4 expression in malignancy represent driver or passenger events in tumorigenesis. We thoroughly studied ERBB4 expression in human colorectal cancer (CRC) using an expression array data set, an immunohistochemistry tumor array and quantitative immunoblot analysis of matched normal/tumor tissue lymphes. We then tested the functional impact of ERBB4 knockdown or over-expression on the transformed phenotype in human CRC cells, as well as in a defined system using mouse colonocytes expressing mutant Apc and Ras. We find that ERBB4 is over-expressed in a significant proportion of human CRC, and that it enhances the transformed phenotype in colonocytes both in vitro and in vivo. These findings provide evidence that increased expression is functionally relevant and that ERBB4 over-expression promotes tumorigenesis.

Materials and methods

Microarray experiments, human tissues and microarray platform

The protocols and procedures for this study were approved by the institutional review boards at University of Alabama-Birmingham Medical Center, Vanderbilt University Medical Center, Veterans Administration Hospital (Nashville, TN) and H. Lee Moffitt Cancer Center, and written informed consent was obtained from each subject per institutional protocol. Representative sections of fresh tissue specimens were flash frozen in liquid nitrogen and stored at −80°C until RNA isolation. Quality assessment slides were obtained to verify the diagnosis of cancer or normal adjacent mucosa. Stage was assessed by American Joint Commission on Cancer slides were obtained to verify the diagnosis of cancer or normal adjacent mucosa. Stage was assessed by American Joint Commission on Cancer.

Protein lysates and western blot analysis

Whole protein lysates from flash-frozen human tumors, patient-matched uninvolved normal biopsies or xenografts grown in nude mice were prepared in standard radioimmunoprecipitation assay buffer with protease and phosphatase inhibitor cocktails (Sigma Corp.) and homogenized with a TissueLyser LT (Qiagen). Cell line lysates were prepared by scraping cells in radioimmunoprecipitation assay buffer with inhibitors and extracting on ice for 15 min. Thirty microgram of protein/sample in Laemmli buffer was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to quantitative immunoblot using the Odyssey imaging system (Li-Cor).

Immunohistochemistry

The tissue micro-array (TMA) was constructed on a manual Beecher arrayer and consisted of triplicate 1 mm cores from 106 primary colorectal carcinomas (see Table 1). Cases were chosen to include a variety of CRC subtypes (mucinous, medullary and signet ring cell carcinomas) and all four stages. Ten cores of normal colonic mucosa were included as controls. The cases have extensive associated pathologic and clinical data, including overall survival. Slides were stained by standard immunohistochemistry with heating in 1 mM of ethylenediaminetetraacetic acid for antigen retrieval and methyl green counterstain. Scoring: ERBB4-stained CRC-TMAs were scored using the Ariol Platform Image Analysis software (Genetic) using tissue-wide binding metrics applied in an unbiased fashion generating a foci count per core (ERBB4 score).

Cell lines

HT-29, Caco-2 and HCT116 cells were obtained from The American Type Culture Collection. HCA-7, LIM2405, LIM1863 and LIM1215 human CRC cell lines (15) and the IMCE-Ras mouse colonocyte line expressing v-Ha-Ras and mutant Apc (16) were a kind gift from Dr Robert Whitehead who directed the Vanderbilt University Digestive Disease Research Center Novel Cell Line Core. The core characterizes, maintains and authenticates these lines. Cell lines were cultured for 6 months in our laboratory before reverting to a frozen stock vial obtained from either The American Type Culture Collection (ATCC) or the Vanderbilt Novel Cell Line Core. CRC lines were grown in RPMI 1640 with 5% fetal bovine serum, 100 U/ml pen-strep and insulin transferrin selenium supplement (BD Biosciences). IMCE-Ras cells were cultured at 33°C in RPMI 1640 with 5% fetal bovine serum, 5 U/ml mouse interferon-γ (Intergen), 100 U/ml pen-strep and insulin transferrin selenium supplement. IMCE-Ras-Vec and IMCE-Ras-ERBB4 cells were generated by stably transfecting IMCE-Ras cells with pcDNA 3.1 (Zeo)-ERBB4 (JM-b, CYT-2 isoform). Continued presence of the ‘immortal’ SV40 large T antigen construct, Ras expression and ErbB4/vector expression were periodically monitored in these cells by PCR.

Serum-free survival and soft agar colony forming assays

Serum-free survival of IMCE-Ras cells was determined by growing cells to confluence then shifting to non-permissive conditions (37°C in RPMI 1640 with 100 μl pen-strep only) and counting cells every 24 h using an x20 microscope objective. IMCE-Ras-ERBB4 cells were grown on 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium-based colorimetric assay (Promega). Anchorage-independent growth was tested by embedding cells in a 0.35% Noble agar (Sigma)/growth medium gel overlaid on a 0.5% agar/growth medium support; colonies were counted after 14 days.

Nude mouse tumor studies

Eight-week-old athymic mice (Foxn1 nu/nu) were purchased from Harlan Sprague Dawley. IMCE-Ras-Vec or IMCE-Ras-ERBB4 were grown on plastic culture dishes according to standard culture techniques. 2 × 106 cells in 200 μl of phosphate-buffered saline were injected into the dorsal flank...
Table 1. Tumor TMA (n = 104)

<table>
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<th>Sex (% male)</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
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<td>50 (48.1%)</td>
<td>9 (8.6%)</td>
<td>23 (22.1%)</td>
<td>19 (18.5%)</td>
<td>3 (1.9%)</td>
</tr>
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<td>26 (25.0%)</td>
<td>12 (11.5%)</td>
<td>13 (12.5%)</td>
<td>19 (18.2%)</td>
<td>84 (80.8%)</td>
</tr>
<tr>
<td>1 (1.0%)</td>
<td>10 (9.6%)</td>
<td>17 (16.2%)</td>
<td>15 (14.3%)</td>
<td>712 (67.6%)</td>
</tr>
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</table>

(17). Results are reported as mean tumor volume [V = (width^2 x length)/2]. About 5 μm sections of formalin-fixed tumors were prepared and stained with hematoxylin and eosin. All in vivo procedures were carried out in accordance with protocols approved by the Vanderbilt Institutional Animal Care and Use Committee.

Intestinal enteroids

Epithelial-only intestinal minigut cultures from freshly isolated mouse intestinal crypts were established essentially as described previously (18), from ErbB4^+/lox^ and ErbB4^lox/lox^; Villin-Cre mice (19,20) on a C57BL/6 background. Crypts were isolated by calcium chelation and mechanical agitation, then embedded in Matrigel (BD Biosciences) droplets with EGF, Noggin and R-Spondin. Once established, cultures were broken up by passing twice through a 27 gauge needle and replated. Percentage of crypts forming stem cell-rich spheroids (21–23) after 24 h were scored. After 14 days of culture, enteroids were harvested, RNA was extracted and Lgr5 expression was determined by quantitative real-time PCR (SYBR green chemistry, 2^-ΔΔCT method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference). Primers used were as follows: Lgr5-forward, ACCTTGGCTAGTATGCAATGC; Lgr5-reverse, TCCAAAGGGCTAGTCGTAT; GAPDH-forward, TGCTCAAGGCAAGG TCTATGAAAG and GAPDH-reverse, TGCTGAGCTAAAACACCCAGTC.

Data analysis

Microarray data (Figure 1A) were normalized using the Robust MultiChip Averaging algorithm as implemented in the Bioconductor package Affy as described previously (24–26). Wilcoxon rank sum test was used to determine significance for the normal, adenoma and CRC comparisons. In vitro data and cell line analyses represent results of at least four independent experiments. Number of specimens for human tissues and mouse xenografts are described in the Results. Data comparing two groups of parametric data were analyzed by t-test. For TMA analysis, normal and CRC/ERBB4 intensity were grouped into ‘low’ <8000 or ‘high’ ≥ 8000 and a Fisher’s exact test was performed.

Results

ERBB4 levels are increased in colorectal carcinoma

Analysis of the The Cancer Genome Atlas data set (27,28) indicates a survival disadvantage in colorectal carcinoma with ERBB4 alteration (Figure 1A). Although this data set identified only mutations and not gene duplications or deletions, it suggested a role for altered ERBB4 activity in CRC. Thus, we analyzed a combined Moffitt Cancer Center and Vanderbilt Medical Center colon cancer expression array data set containing 250 tumor samples (25). This set includes adenomas as well as cancers classified by stage and normal controls (24). ERBB4 messenger RNA levels were significantly increased in all tumor stages compared with normal, including adenomas, indicating that increased ERBB4 expression is an early, premalignant event (Figure 1B). Gene expression was confirmed by quantitative PCR on a second complementary DNA array data set (Origene) containing paired normal and tumor samples. In 8 of 24 pairs, ERBB4 levels were elevated >2-fold in tumor versus normal (data not shown). We determined that the increase in message was paralleled by an increase in protein levels by two methods: immunoblotting for ERBB4 in a set of matched adjacent normal/CRC samples and immunohistochemical staining for ERBB4 in a 109 sample CRC-TMA. Quantitative western blot analysis for ERBB4, normalized to β-actin, revealed >2-fold ERBB4 expression in tumors versus uninvolved normal tissue from 10 of 23 (43%) pairs examined (Figure 1C and D). It should be noted that the rare tumors with reduced expression (green dots) had no obvious characteristics separating them from the other specimens; this may represent a distinct subgroup of tumors or expression of less-stable ERBB4 isoforms (29). Examination of the ERBB4 staining pattern in the CRC-TMA revealed both membrane (full-length receptor) and diffuse cytoplasmic (suggesting receptor activation and cleavage as well as presence of the 80kDa intracellular fragment) staining in both normal and CRC samples. Similar to the RNA and immunoblot data, quantitative analysis of ERBB4 staining using an unbiased automated approach revealed that 17% of CRC samples had levels of expression higher than any of the control levels (Figure 1E). Collectively, these data argue for increased ERBB4 expression in a subset of CRC samples.

Poorly differentiated human CRC lines express high levels of ERBB4

To develop an in vitro model for understanding the effects of ERBB4 expression in CRC, we screened a panel of CRC cell lines for expression of the receptor by western blot analysis. All lines analyzed had detectable expression of both full-length and ICD forms (Figure 2A). The multiple bands observed were confirmed as specific by peptide competition (data not shown) and may represent posttranslational modification or splice variation. Interestingly, the LIM2405, LIM1863, HCT116 and HCA-7 lines showed more robust full-length receptor expression than Caco-2, HT-29 and LIM1215, suggesting a possible inverse correlation between ERBB4 expression and level of differentiation in the CRC cells (15,30). Soft agar colony forming assays showed that LIM2405 cells (which expressed the highest levels among the lines tested) stably expressing short hairpin RNA to ERBB4 had significantly reduced anchorage-independent growth capacity versus cells expressing a control (non-targeting) short hairpin RNA (Figure 2B).

ERBB4 enhances mouse colon epithelial cell transformation

ERBB4 expression in the non-transformed YAMC cell line (31), while it resulted in formation of a small number of colonies in soft agar (4), did not confer the ability to form tumors in a nude mouse xenograft assay (data not shown), indicating that ERBB4 alone is not sufficient as a ‘single hit’ for tumor establishment in vivo. Thus, to directly test whether ERBB4 expression cooperates with known oncogenic mutations in colonocyte transformation, we generated mouse colon epithelial cells over-expressing ERBB4 on an Apc^min^/v-Ha-Ras background (IMCE-Ras cells (16)). ERBB4 over-expression (Figure 2C) enhanced serum-free survival of these cells in culture on plastic (Figure 2D) and doubled the number of colonies at 2 weeks in a soft agar anchorage-independent growth assay (Figure 2E).

Over-expression of ERBB4 enhances IMCE-Ras tumor growth in athymic mice

Because ERBB4 enhanced anchorage-independent growth in vitro, we hypothesized that it would promote in vivo growth
characteristics in a xenograft tumor model. IMCE-Ras-Vec (empty vector) or IMCE-Ras-ERBB4 (expressing ERBB4) cells were injected into the dorsal flank of athymic ‘nude’ mice. By day 14, the IMCE-Ras-Vec line average tumor volume was 122.4 ± 10.73 mm³. In contrast, the IMCE-Ras-ERBB4 line exhibited almost twice the tumor growth, with average tumor volumes of 230.2 ± 27.32 mm³ (Figure 3). This indicates that ERBB4 is capable of augmenting in vivo tumor growth. Interestingly, linear regression of volume versus ErbB4 protein expression (quantified by western blot) in the xenografts suggests that variability in the tumor sizes correlates with ErbB4 protein levels (Pearson’s $r = 0.786$, $r^2 = 0.609$).

**ERBB4-expressing nude mouse xenograft tumors display elevated cell survival signaling**

To determine whether survival signaling pathways are elevated in ERBB4-expressing xenografts in vivo, tumors derived from IMCE-Ras cells expressing empty vector or ERBB4 were homogenized...
and subjected to western blot analysis. In ERBB4-expressing cells, Akt and EGFR phosphorylation, as well as BCL-2 and COX-2 expression, were elevated (Figure 4) when compared with empty vector-expressing tumors. In contrast, p38 mitogen-activated protein kinase phosphorylation (a marker for cellular stress) and levels of the p85 fragment of poly ADP-ribose polymerase (a marker of apoptosis) were decreased in ERBB4-expressing tumors.

**ErbB4-null in vitro minigut cultures have reduced Lgr5 expression and impaired spheroid formation**

Tumorigenesis, in many ways, recapitulates stem cell biology. Recently, an ex vivo system for studying stem cell function has been developed using primary crypt epithelial cells grown in 3D culture. To assess the requirement for ERBB4 in stem cell function, we used this well-characterized intestinal enteroid model for intestinal tumors (32,33). To assess the role of ERBB4 in regulating Lgr5+ stem cell growth, we performed quantitative PCR analysis of these primary tissue-derived enteroids. We found a 75% reduction (P < 0.001) of Lgr5 in the ErbB4-null cultures (Figure 5B). Thus, loss of ERBB4 impairs the activity of Lgr5+ stem cells, which have been implicated in intestinal tumorigenesis.

**Discussion**

Expression differences in ERBB family members have been reported in multiple solid tumor malignancies, and therapies directed against EGFR (ERBB1) and ERBB2 are currently in clinical use in breast cancer (34). Compared with EGFR and ERBB2, however, the relevance of ERBB4 as a therapeutic cancer target, especially in the colon, is largely unexplored. Over-expression is observed in endometrial (35) and non-small cell lung cancers (36), and correlates with poor response to therapy for bone sarcoma (37) and with ovarian cancer resistance to cisplatin (11). On the other hand, data from several other tumor types such as transitional cell carcinoma of the bladder (38) and prostate cancer (39) show either no correlation between ERBB4 levels and tumor behavior, or even an association between receptor expression and good prognosis. Interestingly, studies of ERBB4 in breast cancer have yielded a contradictory literature. Some clinical reports indicate a clear correlation between ERBB4 levels and breast cancer incidence (40) or aggressive tumor behavior, whereas other investigators observe an association between ERBB4 expression and favorable breast cancer outcome (3). Clearly, the mechanisms of ERBB4 involvement in carcinogenesis have not been fully delineated. Determining the roles of this receptor and its downstream targets in different tumor types will be necessary for developing overall models of ERBB4 in carcinogenesis as well as targeted therapies.

In this report, we studied the role of ERBB4 in CRC, testing the hypothesis that altered ERBB4 function influences tumor biology. We first demonstrated elevated levels of ErbB4 messenger RNA in all stages of CRC, including the premalignant adenoma,
indicating that ERBB4 over-expression is an early event in tumorigenesis. Increased expression was confirmed at the protein level via screening of a large CRC-TMA and western blots of matched normal/tumor lysates. Of note, there was heterogeneity in expression which is consistent with prior studies, primarily consisting of immunohistochemical screens, which demonstrated elevated ERBB4 expression in a subset of CRCs (6,41). Unlike our study, however, prior reports did not directly compare tumor to matched normal tissue. Importantly, ERBB4 knockdown in a poorly differentiated CRC cell line impaired anchorage-independent growth, suggesting that ERBB4 contributes to maintenance of malignant phenotypes. In mouse colonocytes with activating mutations in WNT and Ras, we observed an enhancement of anchorage-independent growth in vitro and xenograft establishment in vivo with ERBB4 over-expression. Furthermore, ERBB4 knockout impaired the growth of WNT-dependent intestinal minigut cultures. Together, these data indicate that ERBB4 over-expression can cooperate with activated WNT signaling to enhance the transformed phenotype in mouse and human colonocytes.

The mechanism leading to tumor over-expression of ERBB4 at the RNA and protein level is a key follow-up question for these studies. The Cancer Genome Atlas has provided a wealth of information about the ‘-omics’ of malignancy and examination of ERBB4 in this data set yields several observations. Firstly, non-synonymous mutations in ERBB4 (7.5–11% of the cases) predominate in comparison with amplifications. In fact, in all of the CRC data sets we examined, there is not a single identified amplification or deletion (27,28), suggesting that ERBB4 over-expression is not likely due to gene duplication events. In contrast, the expression may be driven by the microenvironment and occurring at the transcriptional and/or protein stability level. We have shown previously that, in non-transformed colonocytes, ERBB4 expression and activation are strongly induced by inflammatory cytokines (e.g. TNF) in a mechanism likely involving nuclear factor-κB (5). In human CRC cell lines, we have preliminary data (M.A.Schumacher and M.R.Frey, unpublished observations) showing ERBB4 message induction by TNF; furthermore, this response is stronger in lines with relatively low expression (HT-29) versus those with higher levels (HCT116). As almost all tumors are associated with at least some level of local inflammation (42,43), this may point to a mechanism by which ERBB4 levels are elevated in CRC. An additional possibility

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**Figure 3.** ERBB4 enhances tumor growth in athymic mice. $2 \times 10^6$ IMCE-Ras cells expressing empty vector ($n=28$) or ERBB4 ($n=27$) were injected subcutaneously into the dorsal flanks of 8 week athymic ‘nude’ mice. (A) Images of representative tumors from vector- and ERBB4-expressing cells. (B) Quantification of growth achieved by measured tumor dimensions at 14 days is presented as average tumor volume ($V = (\text{width}^2 \times \text{length})/2$) ± standard error. (C) Representative hematoxylin- and eosin-stained sections from vector- or ERBB4-expressing tumors. (D) Linear regression of tumor volume as a function of ErbB4 protein expression determined by quantitative western blot analysis (normalized to actin) of lysates from nine xenografts recovered on necropsy.
Recent studies from the Elenius lab using breast cancer cells have revealed that the HIF-1α hypoxia-inducible factor subunit stabilizes the ERBB4 ICD in the cytoplasm and increases ERBB4 activity. As hypoxia is a common characteristic of tumors, and HIF-1α signaling promotes the transformed phenotype, hypoxia-induced stabilization of ERBB4 protein is an interesting possible second regulatory mechanism. These candidate pathways and their roles in tumor cell biology are the focus of ongoing studies in the laboratory.

Analysis of the Vanderbilt/MCC transcriptome data set via stratification of clinical treatments and outcomes by ERBB4 transcript levels did not reveal a significant influence of ERBB4.
message levels on either of these clinical variables. This is not necessarily surprising, given that over-expression appears to occur early in tumorigenesis (Figure 1B). ERBB4 promotes survival of both non-transformed and transformed colonocytes (4,7,13), and thus, its over-expression may allow for a permissive state in which tumorigenic genomic alterations can occur (48). In support of this concept, our enteroid ‘minigut’ data (Figure 5) showing that loss of ERBB4 impairs both establishment and regeneration of culture upon disruption suggests a role in regulating function of Lgr5+ intestinal stem cells, thought to be an important contributor to intestinal adenoma development (33,49).

Increased activation of cell survival pathways and decreased expression of apoptotic markers in ERBB4-over-expressing xenografts is consistent with our previous in vitro results in non-transformed colonocytes (4,5,7). ERBB4 protects young adult mouse colon epithelial cells from TNF-induced apoptosis, and knockdown of the receptor sensitizes these cells to insult (5). ERBB4 over-expression is associated with increased EGFR activation, PI3K signaling and COX-2 expression (4). We and others also observed increased apoptosis in human CRC cells when ERBB4 was knocked down by small interfering RNA (4,13). Together these results outline an antiapoptotic role for ERBB4 in both normal and transformed cells, raising the possibility that although induction or activation of the receptor in response to injury or inflammation may be an acute protective response (5,7), chronic over-expression can contribute to enhanced EGFR/PI3K signaling and increased COX-2 levels, resulting in a permissive environment for survival and growth of transformed cells.

Functional cooperation between ERBB4 and other family members, including EGFR, has long been assumed, but is not well studied, especially in the gastrointestinal tract. Work from the Threadgill laboratory demonstrated that ERBB3 deletion resulted in loss of ERBB4 expression, suggesting joint regulatory mechanisms (13), and ERBB4 co-expression with other ERBB family members has been noted in some immunohistochemical surveys of CRC (6,41). In this study, ERBB4 over-expression enhanced activation of EGFR and some pathways known to be EGFR targets such as AKT phosphorylation and COX-2 induction (Figure 4). This is consistent with a recent report in the breast cancer literature showing that the 80kDa fragment of ERBB4 enhances EGF-stimulated cell motility (50). Interestingly, we previously showed that, upon ERBB4 over-expression, heregulin-1i promotes EGFR phosphorylation in non-transformed intestinal cells (4) despite not directly binding EGFR, and EGFR knockdown reduced heregulin-1i-stimulated ERBB4 activation. This response is dependent on the specific ERBB4 ligand applied; in contrast with heregulin-1i, neuregulin-4 specifically promotes ERBB4—but not EGFR, ERBB2 or ERBB3—phosphorylation in intestinal cells (7,51). Although beyond the scope of this study, a detailed investigation of the different ligand-specific partnerships between ERBBs in colonocytes, and whether their interactions are at the level of heterodimerization or downstream signaling interaction, represents an important next step toward untangling ERBB4 biology in the normal colon and CRC.

An important biochemical aspect to ERBB4 that is unique amongst the ERBB family is the expression of splice variants that either do (or do not) bind PI3K directly (CYT-1 versus CYT-2 cytoplasmic domain variants) or that are (or are not) subject to two-step proteolytic cleavage (JM-a versus JM-b juxtamembrane domain variants). In some tumor types, selective isoform expression appears to be important for determining whether ERBB4 is tumorigenic or not. For example, in breast cancer, cytoplasmic/nuclear ERBB4 staining (presumably reflecting presence of the cleavable JM-a isoform) is generally associated with poor prognosis and more aggressive tumor features, whereas exclusively membrane staining (i.e. non-cleavable JM-b isoform) is not (52,53). The probe set analyzed in Figure 1B does not distinguish between messenger RNA for the different isoforms, but at the protein level, we observe robust expression of the 80kDa cleavage product (Figure 1C and I). This is consistent with the findings from breast cancer. On the other hand, our previous work showed that ERBB4 over-expression enhanced PI3K activation, COX-2 expression and cell survival regardless of which isoform was used (4,5). Several potential explanations for this apparent discrepancy are currently under investigation in our lab. First, ERBB4 is known to promote its own expression in some systems (54), and it is possible that ectopic over-expression of a non-cleavable isoform in colonocytes also induces endogenous cleavable isoform production. Additionally, ERBB4 likely induces cell survival through more than one pathway; for example, the antiapoptotic response to high levels of over-expression and heterodimerization with EGFR depend on COX-2 activity (4), whereas modest, selective ERBB4 activation with its specific ligand NRG4 protects cells from cytokine-induced death in a COX-2 independent manner (7). Further study is required to distinguish between these two possibilities and thus define the potential utility of isoform-specific targeting approaches as have been proposed for some cancers (55).

In summary, our data show that the ERBB4 receptor tyrosine kinase is over-expressed at both message and protein levels in human CRC. ERBB4 loss impaired transformation of human CRC cells and the ability of mouse intestinal stem cells to grow in culture; in contrast, ERBB4 over-expression enhanced tumorigenicity of transformed mouse colonocytes. High ERBB4 levels were associated with activation of EGFR and PI3K pathways as well as COX-2 expression. Together, these results suggest that ERBB4 may be a valid therapeutic target in colorectal and perhaps other epithelial-based malignancies.

Supplementary material
Supplementary material is available with this article: Figure 1, containing un-cropped blot images for all results can be found at http://carcin.oxfordjournals.org/

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