The Receptor Tyrosine Kinase EPHB4 Has Tumor Suppressor Activities in Intestinal Tumorigenesis

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Abstract

Colorectal cancer is the second cause of cancer-related death in the western world, and although the genetic and molecular mechanisms involved in the initiation and progression of these tumors are among the best characterized, there are significant gaps in our understanding of this disease. The role of EPHB signaling in colorectal cancer has only recently been realized. Here, we use animal models to investigate the role of EphB4 in intestinal tumorigenesis. Modulation of EPHB4 levels in colon cancer cell lines resulted in significant differences in tumor growth in a xenograft model, with low levels of EPHB4 associated with faster growth. In addition, using a genetic model of intestinal tumorigenesis where adenomatous polyposis coli (Apc) mutations lead to initiation of the tumorigenic process (Apc<sup>min</sup> mice), we show that inactivation of a single allele of EphB4 results in higher proliferation in both the normal epithelium and intestinal tumors, significantly larger tumors in the small intestine, and a 10-fold increase in the number of tumors in the large intestine. This was associated with a 25% reduction in the lifespan of Apc<sup>min</sup> mice (P < 0.0001). Gene expression analysis showed that EphB4 mutations result in a profound transcriptional reprogramming, affecting genes involved in cell proliferation, remodeling of the extracellular matrix, and cell attachment to the basement membrane among other functional groups of genes. Importantly, in agreement with the expression profiling experiments, using an in vitro assay, we show that loss of EPHB4 in colon cancer cells results in a significantly increased potential to invade through a complex extracellular matrix. Collectively, these results indicate that EphB4 has tumor suppressor activities and that regulation of cell proliferation, extracellular matrix remodeling, and invasive potential are important mechanisms of tumor suppression. [Cancer Res 2009;69(18):7430–8]

Introduction

Colorectal cancer is one of the leading causes of cancer-related death in the western world and accounts for ~1 million new cases and ~500,000 deaths every year worldwide. At the molecular level, the constitutive activation of Wnt signaling is one of the hallmarks of colorectal cancer. Wnt activation most frequently occurs through Apc mutations, preventing the phosphorylation of the key coactivator β-catenin, which accumulates in the nucleus, binds to TCF/LEF transcription factors, and activates the promoter of a large number of mitogenic genes, such as c-MYC, cyclin D1, and several EPHB receptors (1–4). Cell proliferation and positioning in both normal and transformed intestinal epithelium are largely controlled by Wnt signaling through EPH signaling cascades. EPH receptors constitute the largest receptor tyrosine kinase family with at least 16 different receptors identified, and EPHs are grouped into A and B subtypes according to sequence homology and ligand binding specificity (5). EPHB2 and EPHB3 have been shown to regulate both proliferation in the intestinal crypts and positioning of cells of different lineage within the normal intestinal epithelium (3, 6). Although EPHB2/3 knockout mice do not develop tumors, the oncogenic process is substantially accelerated upon tumor initiation by adenomatous polyposis coli (Apc) inactivation (4). EPHB4 expression has been shown to be frequently lost in advanced human colorectal tumors and we have recently reported that low tumor EPHB4 levels are associated with poor prognosis of colorectal cancer patients, and modulation of EPHB4 levels in colorectal cancer cell lines interfered with the long-term clonogenic potential of these cells (4, 7). However, EPHB4 has oncogenic activities in a variety of tumor types, including prostate, breast, endometrium, mesothelioma, head and neck, bladder, and ovarian cancer (8–14), and after a recent study reported a possible oncogenic effect of EPHB4 (15), the functional role of EPHB4 in colorectal tumors remains to be elucidated.

In this study, we investigate the role of EPHB4 in colorectal tumorigenesis using animal models. We used a xenograft approach to show that restoring EPHB4 levels in deficient colon cancer cells leads to significantly smaller tumors, whereas inactivation of EPHB4 signaling in cell lines with high levels results in faster tumor growth. In addition, loss of a single allele of EphB4 was found to result in a 25% reduction of the lifespan of Apc<sup>min</sup> mice where intestinal tumorigenesis is initiated by Apc inactivation. A 2-fold reduction in EphB4 expression was observed in the normal epithelial crypts and intestinal tumors. The reduced lifespan of EphB<sup>+/−</sup> mice compared with EphB<sup>+/+</sup> animals was associated
with higher proliferation in both the normal epithelium and intestinal tumors as well as a significantly increased tumor burden. This was linked with the transcriptional reprogramming of multiple growth factors and genes involved in extracellular matrix remodeling. Importantly, the role of EPHB4 in the capacity of colon cancer cells to invade through the extracellular matrix was directly shown using an in vitro assay. Collectively, these results indicate that EPHB4 has tumor suppressor activities in intestinal tumorigenesis and show that tumor suppression is associated with the control of cell proliferation, extracellular matrix remodeling, and invasive potential.

Materials and Methods

Cell lines. We have previously assessed EPHB4 levels in a panel of colorectal cancer cell lines and found that SW837 and HT29 have low and high relative levels of EPHB4, respectively (7). SW837 cells were stably transfected with a construct expressing full-length EPHB4 (16) or the corresponding empty vector control (pcDNA3.1; Invitrogen). HT29 cells were stably transfected with pEGFP-N2 (Clontech) or pEPHB4ΔC-EGFP, a construct coding for a chimeric EPHB4 protein where the kinase domain has been substituted by enhanced green fluorescent protein (EGFP). This protein has been shown before to function as a dominant negative (17).

Western blot. Extracts of total protein (50 μg) in radioimmunoprecipitation assay buffer were loaded on a 10% acrylamide gel. After gel electrophoresis, proteins were transferred to a nitrocellulose membrane and probed with anti-EPHB4 (1:200; clone 3D7G8; Zymed Laboratories), anti-EGFP (1:1,000; Clontech), or anti-β-actin (1:1,000; clone AC74; Sigma) as described previously (7).

Xenograft model. Six athymic nude-Foxn1^nu mice (Harlan) were injected s.c. with 1.5 × 10^6 SW837-pcDNA3.1 (left flank) and SW837-pcDNA3.1-EPHB4 (right flank) cells resuspended on growth medium. The same experimental setup was carried out for the HT29 pEPHB4ΔC-EGFP derivative line and the corresponding empty vector control. Tumor size was measured using a caliper three times per week for a total of 6 weeks. Tumor volume was calculated with the formula: \( V = (L \times W^2) / 2 \times 0.5 \), where \( L \) is the length and \( W \) is the width of a xenograft.

Mouse knockout strains. The C57BL/6/J-Apc^min/+ strain was obtained from The Jackson Laboratory. These mice carry a heterozygous mutation in Apc. EPHB4/LacZ mice carry an EPHB4 allele in which the signal sequence has been deleted and a tau-lacZ indicator gene has been fused in-frame with the initiation codon, preventing membrane insertion of the receptor (18). Male Apc^min/+;EphB4+/+ mice were crossed with female Apc^min−/−;EphB4+/+ mice and the resulting Apc^min−/−;EphB4+/+ offspring were used in the experiments described.

Immunohistochemistry. Eighteen-week-old mice were sacrificed, the small and large intestines were dissected and opened longitudinally, and tumor size and number were scored. The tissue was then fixed with 10% formalin and embedded in paraffin. Antigen retrieval was carried out with the RNasea Micro Kit (Qiagen) and reverse transcribed to cDNA using the cDNA Archive Kit (Applied Biosystems). EPHB4, IGFBP5, MMP2, and Col5a2 levels were assessed by real-time PCR using SYBR Green Master Mix (Applied Biosystems) and 18S rRNA was used as a standardization control. Primer sequence is available in Supplementary Fig. S3. Relative mRNA levels were assessed using the 2^(-ΔΔCt) method as described before (21, 22).

Microarray experiments. Fresh tumors from the jejunum were dissected and total RNA was extracted as described above. Equal amounts of RNA from three different tumors of 18-week-old mice were pooled and processed for hybridization on Affymetrix Mouse 430 2.0 chips (21). Tumor pools from two Apc^min−/−;EphB4−/− and two Apc^min−/−;EphB4+/+ mice were hybridized independently. The expression values obtained from all the hybridizations were normalized using dChip software (23). Functional group enrichment analysis was used to compare the number of genes present in the Affymetrix 430 2.0 chip in each one of the Gene Ontology (24) categories (cellular component, molecular function, and biological process) and the number of genes in each one of these categories in the list of 183 genes differentially expressed in tumors from Apc^min−/− mice that are wild-type or heterozygous for EphB4. This analysis was implemented using L2L software (25) and a level of significance of \( P < 0.05 \) (binomial test) was used. We performed a Functional Network Analysis of the 183 differentially expressed genes found in the microarray experiments using the Web-interface MouseNet version 1 (26). The resulting network was exported to Cytoscape 2.6.0 for graphical representation (27).

Results

EPHB4 regulates the growth of colon cancer cells. We have reported previously that EPHB4 levels regulate the clonogenic potential of colorectal cancer cells (7). Here, we used a xenograft model to evaluate the role of EPHB4 on the growth of human colon cancer cells. We stably transfected a colon cancer cell line expressing relative high levels of EPHB4 (HT29) with a construct containing the human EPHB4 extracellular and transmembrane domain and EGFP instead of the kinase domain (pEPHB4ΔC-EGFP; Fig. 1A). This protein has been shown to function as a dominant-negative form of EPHB4 (EPHB4-DN; ref. 17). We then injected s.c. 1.5 × 10^6 cells of the empty vector control HT29-EGFP-EV line (left flank) or HT29-EPHB4-DN cells (right flank) in six athymic nude mice. Xenograft growth was monitored three times per week for a total of 6 weeks. Overexpression of the dominant-negative EPHB4 was shown to result in significantly larger tumors compared with control cells (Fig. 1B). In addition, using a cell line with low relative levels of EPHB4 (SW837), we engineered an isogenic derivative line that overexpressed this receptor (Fig. 1C). When grown as a xenograft in nude mice, overexpression of EPHB4 resulted in significantly slower tumor growth (Fig. 1D). EPHB4 has been reported previously to modulate angiogenesis in normal and tumor tissues (18, 28). However, immunohistochemical staining of

http://rsweb.nih.gov/ij/
the endothelial markers CD34 and CD105 (endoglin) did not reveal significant differences in the vasculature of the xenografts of the isogenic cell lines where EPHB4 levels were altered (Supplementary Fig. S1A-D). Together, these results suggest that the lower levels of EPHB4 frequently observed in colorectal tumors (4, 7) may contribute to tumor progression.

**EphB4 inactivation results in shorter survival of Apc\(^{min/+}\) mice.** To further investigate the role of EphB4 on intestinal tumorigenesis, we used a genetic mouse model carrying an inactivated EphB4 allele (18). Homozygous mutations in this receptor cause embryonic lethality, whereas heterozygous animals are viable and have no gross abnormalities (18). Because no tumors have been reported in the gastrointestinal tract of EphB4\(^{+/+}\) mice, we decided to initiate the tumorigenic process introducing a mutant allele of Apc. Heterozygous Apc mutant animals (Apc\(^{min/+}\)) developed multiple tumors within the gastrointestinal tract and had a median lifespan of 199 days (Fig. 2). Introduction of a single mutant allele of EphB4 on the Apc\(^{min/+}\) background shortened the lifespan of these animals by \(\sim 25\%\) compared with EphB4\(^{+/+}\) littermates (median survival of 149.5; \(P < 0.0001\), log-rank test; see Fig. 2), suggesting that EphB4 inactivation accelerates the tumorigenic process in these animals.

**EphB4 inactivation results in larger tumors in the small intestine and higher tumor number in the large intestine.** To understand the underlying cause of the shorter survival observed in Apc\(^{min/+}\);EphB4\(^{+/+}\) animals compared with Apc\(^{min/+}\);EphB4\(^{+/+}\), we investigated the number, size, and location of the tumors found in animals sacrificed at age 18 weeks. There were no significant differences in the number of tumors found in the small intestine (22.1 \(\pm\) 11.8 and 26.1 \(\pm\) 12.7 in EphB4\(^{+/+}\) and EphB4\(^{+/+}\), respectively; \(P = 0.48\); Fig. 3A). However, the average size of the tumors found in Apc\(^{min/+}\);EphB4\(^{+/+}\) mice was \(\sim 50\%\) larger than the tumors found in Apc\(^{min/+}\);EphB4\(^{+/+}\) mice (1.59 \(\pm\) 0.44 and 1.06 \(\pm\) 0.18 mm, respectively; \(P = 0.0002\); Fig. 3B). The majority of the tumors observed in Apc\(^{min/+}\) mice were located in the jejunum. However, mutation of one allele of EphB4 in Apc\(^{min/+}\) animals resulted in a shift toward more proximally located tumors (\(P = 0.0007\), Fisher’s test). Whereas only 20% of the tumors in

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**Figure 1.** EPHB4 levels regulate tumor growth in a xenograft model. HT29 cells (1.5 \(\times\) 10\(^6\)) stably transfected with a dominant-negative form of EPHB4 (EPHB4-\(\Delta C\)-EGFP) or the corresponding empty vector control (A) were s.c. injected in the flanks of immunocompromised nude mice. Introduction of the dominant-negative EPHB4 (dashed line) resulted in significantly increased tumor growth (B). SW837 cells were stably transfected with a vector expressing full-length EPHB4 or the corresponding empty vector control (C). Reintroduction of EPHB4 in SW837 cells (dashed line) resulted in significantly slower tumor growth (D).
EphB4+/− mice were located in the duodenum, 35% of the tumors in EphB4+/− were found in the duodenum. In addition, whereas the large intestine of Apc<sup>min/−;EphB4+/−</sup> animals had only an average of 0.25 macroscopically visible tumors per mouse, Apc<sup>min/−;EphB4+/−</sup> mice had 2.4 tumors on average (9.6-fold increase; Fig. 3C and D). The larger tumors found in the small intestine and the higher tumor frequency in the large intestine are likely to contribute to the shorter survival of the Apc<sup>min/−;EphB4+/−</sup> compared with the Apc<sup>min/−;EphB4+/+</sup> counterpart.

**EphB4 levels, proliferation, apoptosis, and differentiation.**

We used immunohistochemical staining to evaluate the effects of EphB4 mutations in the levels of expression of EphB4 in the normal and transformed intestinal epithelium of Apc<sup>min/−</sup> mice. Rigorous quantification of EphB4 immunostaining showed that targeted deletion of one allele of EphB4 led to a 52% reduction in the levels of expression of EphB4 in the proliferative compartment of the normal small intestine (P = 0.02, Student’s t test; Fig. 4A and B). To further investigate the underlying mechanisms responsible for the shorter survival and higher tumor burden in Apc<sup>min/−;EphB4+/−</sup> mice relative to the Apc<sup>min/−;EphB4+/+</sup> counterpart, we studied the effects on differentiation, apoptosis, and proliferation in the normal intestinal epithelium. Although other EphB receptors have been reported to control lineage-specific cell sorting within the intestinal crypts (3), no differences in the number or localization of goblet cells (Alician blue positive; Supplementary Fig. S2A and B), Paneth cells (lysozyme positive; Supplementary Fig. S2C and D), or enteroendocrine cells (Grimelius staining; Supplementary Fig. S2E and F) were observed in the intestinal epithelium of Apc<sup>min/−;EphB4+/−</sup> and Apc<sup>min/−;EphB4+/+</sup> mice. In addition, no differences were observed in the number of apoptotic cells that were positive for active caspase-3 or terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (data not shown). In the epithelial lining of the normal intestine, cell division is confined to the crypt compartment. We assessed proliferation in the small intestinal crypts by scoring the number of cells that were positive for immunostaining with PCNA, a well-established proliferative marker (Fig. 5). The number of proliferating cells was significantly higher in Apc<sup>min/−</sup> mice that are heterozygous for EphB4 compared with EphB4 wild-type animals (18.3% increase; P = 0.03, Student’s t test; Fig. 5A and C).

The histology of a total of 101 intestinal tumors was microscopically examined. They presented as sessile or plaque-like lesions. The vast majority of these tumors were adenomas with low-grade (55 of 101) and high-grade (46 of 101) dysplasia. Six of the examined tumors were intramusosal adenocarcinomas with lamina propria invasion and one of them showed evidence of vascular invasion. No significant differences were observed in the type of tumors in Apc<sup>min/−;EphB4+/−</sup> mice that were EphB4 wild-type or heterozygous. As reported previously (4), the high levels of EphB4 expression observed in the proliferating cells within the normal crypts were maintained in the adenomatous lesions of Apc<sup>min/−;EphB4+/−</sup> mice (Fig. 4C). Here, we show that the tumors of Apc<sup>min/−;EphB4+/−</sup> mice have reduced EphB4 protein levels (Fig. 4C and D). In good agreement, a 51% reduction in EphB4 mRNA levels (P = 0.0019, Student’s t test) was also observed in tumors from EphB4 heterozygous mice as assessed by quantitative reverse transcription-PCR. To study the underlying mechanisms leading to smaller intestinal tumors, we investigated the effects of the reduced EphB4 levels on apoptosis, vascularization, and proliferation. No changes were observed in the number of apoptotic cells (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling or active caspase-3 positive) in the tumors of Apc<sup>min/−;EphB4+/−</sup> mice compared with tumors in Apc<sup>min/−;EphB4+/+</sup> animals (data not shown). In addition, immunostaining...
with the endothelial marker CD34 did not reveal differences in the vascularization of the small intestinal tumors from Apc\textsuperscript{min/+} mice that are wild-type or heterozygous for EphB4 (Supplementary Fig. S1E and F). However, when assessed as the number of PCNA-positive cells, the loss of a single allele of EphB4 led to a significant increase (33%; \( P = 0.02 \), Student’s \( t \) test) in the number of proliferating cells (Fig. 5B and D). The higher proliferative rates observed in the tumors from Apc\textsuperscript{min/+} mice that are EphB4 heterozygous are in good agreement with the 50% increase in tumor size observed.

**Transcriptional reprogramming imposed by EphB4 inactivation.** To gain further insight into the molecular mechanisms underlying the tumor suppressor activity observed for EphB4 in the intestine of Apc\textsuperscript{min/+} mice, we used oligonucleotide microarray analysis to assess differences in the expression levels of >39,000 transcripts in tumors from Apc\textsuperscript{min/+} mice that are EphB4 wild-type or heterozygous for EphB4. To assess the level of background variability between hybridizations, we examined the mean fold difference of 78 housekeeping genes (29) in tumors from EphB4+/+ and EphB4+/−/C0 animals and was found to be 1.1 ± 0.1 (mean ± SD). The 183 genes with >2-fold expression difference in tumors from EphB4 wild-type and heterozygous mice were considered to be differentially expressed. This cutoff value is >20 times the SD observed in the set of 78 housekeeping genes used to define the baseline variability. Seventeen of the 183 differentially expressed genes were represented by more than one probe set showing excellent agreement between independent expression measurements for the same gene (Pearson’s correlation \( R = 0.94; P < 0.0001 \)). Of the 183 genes differentially expressed, 21 had reduced expression and 162 elevated expression in tumors from Apc\textsuperscript{min/+} mice that were EphB4 heterozygous compared with Apc\textsuperscript{min/+}/EphB4+/−/C0 animals (Supplementary Table S1), indicating a global inhibitory role imposed by EphB4 in intestinal tumors. Quantitative real-time reverse transcription-PCR was used to confirm the expression differences observed in the microarray experiments for four genes (EphB4, Igfbp5, Mmp2, and Col5a2). An excellent correlation was observed between microarray and quantitative reverse transcription-PCR assessments of gene expression (\( R = 0.99; P = 0.003 \); Supplementary Fig. S3).

Activation of the Wnt signaling pathway is one of the hallmarks of intestinal tumorigenesis. Loss of EphB4 in the normal epithelium and small intestinal tumors did not result in differences in Wnt signaling as assessed by the absence of expression differences in a list of 20 well-characterized β-catenin/TCF4 target genes (fold difference <2; Supplementary Table S2), β-catenin immunostaining (Supplementary Fig. S4A-D), and direct assessment of β-catenin/TCF4 transcriptional activity using transient transfection of a reporter construct in colon cancer cells with different levels of EphB4 (Supplementary Fig. S4E). Previous studies have investigated the transcriptional changes leading to the transition
from adenoma to carcinoma in intestinal tumors of Apc<sup>min/+</sup> mice and identified differences in nine key genes (30). Remarkably, an excellent correlation ($R = 0.96; P < 0.003$) was observed between the fold expression difference observed for these genes in the adenoma-to-carcinoma transition and in tumors from Apc<sup>min/+</sup> mice that were wild-type or heterozygous for EphB4 (Supplementary Fig. S5; Supplementary Table S3). In addition, there were a significant number of growth factors up-regulated in tumors from EphB4<sup>+/-</sup> mice compared with EphB4<sup>+/+</sup> animals, including insulin-like growth factor-I, connective tissue growth factor, fibroblast growth factor-7, amphiregulin, epiregulin, discoidin domain receptor tyrosine kinase 2, and platelet-derived growth factor receptor (see Supplementary Table S1). The transcriptional changes observed in tumors from Apc<sup>min/+;EphB4+/-</sup> mice are likely to be responsible for the increased proliferation found in these tumors. Overall, these results indicate that inactivation of a single copy of EphB4 leads to transcriptional changes that favor tumor progression.

Functional group analysis and interactome analysis identified a subset of genes involved in extracellular matrix reorganization, accounting for >28% (52 of 183) of the genes differentially expressed (Supplementary Fig. S6; Supplementary Tables S4-S6). Among these genes, there were 12 different types of collagen (Col15a1, Col18a1, Col1a1, Col1a2, Col3a1, Col4a1, Col4a2, Col5a1, Col5a2, Col6a2, and Col6a3), 3 matrix metalloproteinases (MMP2, MMP10, and MMP13), as well as other proteins involved in remodeling of the extracellular matrix such as decorin, biglycan, lysyl oxidase-like 2, lumican, and Sparc (see Supplementary Table S1). These results strongly suggested the possibility that EphB4 may regulate the capacity of intestinal tumor cells to invade through the extracellular matrix.

**EPHB4 regulates the migration/invasion of colon cancer cells.** To test the hypothesis that reduced EPHB4 levels favor
migration/invasion, we used isogenic HT29 colon cancer cells expressing high levels of EPHB4 and a derivative line stably transfected with a dominant-negative form of EPHB4 (EPHB4ΔC-EGFP; see Fig. 1C). This system models the reduced EPHB4 expression observed in tumors from Apc<sup>min/−</sup>;EphB4<sup>+/−</sup> mice compared with tumors from Apc<sup>min/−</sup>;EphB4<sup>+/+</sup> animals (see Fig. 4C-E) and in human tumors. We used a Matrigel invasion assay to show that reduced EPHB4 signaling resulted in a 5.6-fold increase in the number of invading cells (Fig. 6A). Moreover, overexpression of EPHB4 in SW837 cells led to a 2.5-fold reduction of the invasive potential of these cells (Fig. 6B). These results suggest that the profound expression reprogramming observed in intestinal tumors from EphB4<sup>+/−</sup> mice compared with the wild-type counterpart leads to an increased capacity of the tumor cells to invade/migrate through the extracellular matrix surrounding the tumor.

**Discussion**

EPHB4 plays important roles during embryogenesis and maintenance of normal homeostasis of different adult tissues such as the brain and the vasculature (5). In addition, the expression of EPHB4 has been reported to be deregulated during tumorigenesis in different tissues. Most of the reports point at EphB4 being overexpressed in several tumor types such as prostate, mesothelioma, head and neck, bladder, and ovarian cancer, and elevated levels are linked to a protumorigenic role of this receptor (8, 10–13). However, conflicting results have been reported for EPHB4 in breast tumors, where it may have tumor suppressor activity (9, 31). Although the tumor suppressor effects of other EPHB receptors have recently been shown (4, 6, 32), the possible role of EPHB4 in intestinal tumorigenesis remains unclear. Some studies have reported EPHB4 overexpression in colorectal tumors (33, 34) and the elevated EPHB4 levels have been suggested to confer a growth advantage to colon tumor cells (15). Moreover, EPHB4 targeting has been suggested to be of therapeutic value in colorectal tumors (15). However, this is in disagreement with the results from other studies showing that EPHB4 expression is frequently lost in colorectal tumors (4, 7). Moreover, the loss of EPHB4 expression is associated with tumor dissemination and reduced patient survival (4, 7). Animal models constitute a powerful tool to elucidate the role of cancer genes in the tumorigenic process in vivo. Here, we show that inactivation of a single allele of EphB4 is sufficient to cause a 25% reduction in the lifespan of mice where the tumorigenic process is initiated by heterozygous Apc mutations. We show that the reduced lifespan of EphB4<sup>+/−</sup> animals is associated with increased proliferation, larger tumors in the small intestine, and a 9.6-fold increase in the number of tumors in the large intestine of these mice, indicating a tumor suppressor function for EphB4 in this organ.

Several EPHB receptors, including EPHB2, EPHB3, and EPHB4, have been reported to be direct transcriptional targets of β-catenin/TCF and EphB2 and EphB3 signaling has been shown to be important for maintaining active proliferation in the crypts of the intestinal epithelium (3, 6). Abrogation of EphB2/EphB3 signaling by targeted inactivation in murine models results in ~50% reduction of the number of proliferating cells in normal intestinal crypts and adenomas observed in Apc<sup>min/−</sup> mice (6). In this study, we observed significantly increased proliferation in the normal epithelium and small intestinal adenomas of Apc<sup>min/−</sup> mice that are heterozygous for EphB4 compared with EphB4 wild-type animals. Therefore, unlike other EphB receptors, disruption of EphB4 signaling seems to promote tumorigenesis by increasing proliferation.

Previous studies investigating transcriptional changes associated with tumor progression using the Apc<sup>min/−</sup> mouse model identified a small set of genes associated with the transition from adenoma to carcinoma (30). The expression changes induced by the inactivation of a copy of EphB4 in Apc<sup>min/−</sup> mice, which are associated with larger tumor size and shorter animal lifespan, closely resembled the expression changes observed in the adenoma-to-carcinoma transition (see Supplementary Fig. S5; R = 0.96; P < 0.003). The expression changes induced by EphB4 inactivation in Apc<sup>min/−</sup> mice and in the adenoma-to-carcinoma transition include the up-regulation of insulin-like growth factor binding protein 5, which may potentiate signaling through the proliferatory insulin-like growth factor signaling pathway, and the serine peptidase inhibitor Serpine2. In addition, there was a reduction in the expression of S100g (calbindin D9K), which regulates the intracellular levels of calcium, and the transcriptional regulator Sox17, which is a negative regulator of Wnt signaling and is silenced by promoter hypermethylation in most colorectal tumors (35, 36). Also, deletion of a copy of EphB4 leads to a significant shift in the balance

![Figure 6](cancerres.aacrjournals.org) **Figure 6.** EPHB4 regulates cell invasion. A Matrigel invasion assay revealed that interfering with EPHB4 signaling in HT29 cells using a dominant-negative form of EPHB4 resulted in a 5.6-fold higher number of invading cells (A). Conversely, reintroduction of EPHB4 into SW837 cells led to a 2.5-fold decrease of the invasive potential (B). *, P < 0.01 (Student’s t test).
maintaining the proliferative state of the tumor cells with several mitogenic genes being up-regulated, such as PtgS2/ Cox2, Wnt5a, and Igf1, and some counterbalancing antiproliferative genes, such as secreted frizzled-related sequence protein 1. Collectively, the expression reprogramming observed is likely to underlie the increased proliferation, the larger tumor size, and ultimately the shorter animal survival observed in the EphB4+/−/ mice. Despite the larger adenoma size, we did not observe a shift toward a more aggressive phenotype in tumors from EphB4+/−/ mice. This is in good agreement with the observation that the loss of EphB4 is specifically associated with the transition from small to large adenoma in human lesions (4). Whereas >70% of the human small adenomas (<5 mm) retain high levels of EphB4, 90% of the large adenomas (>5 mm) show reduced EphB4 levels compared with the normal proliferating epithelial cells (P = 0.001, Fisher’s test).

A large proportion of the genes differentially expressed in the intestinal tumors from Apcmin/+ mice that are wild-type or heterozygous for EphB4 are involved in the synthesis and remodeling of the extracellular matrix and regulation of cell attachment to the basement membrane. The profound reprogramming caused by heterozygous EphB4 inactivation included the coordinate up-regulation of 12 different types of collagen as well as multiple enzymes involved in remodeling of the extracellular matrix such as several matrix metalloproteinases (MMP2, MMP10, and MMP13), decorin, biglycan, lysyl oxidase−like 2, lumican, and Sparc (see Supplementary Table S1). Here, we show that EphB4 modulates the capacity of colon cancer cells to invade through a complex extracellular matrix. HT29 cells transfected with a dominant-negative receptor tyrosine kinase EphB4 in mice compared with the wild-type counterpart and may contribute to explain both the transition from small to large adenoma associated with the loss of EphB4 (4) and the poor prognosis of patients with low tumor levels of EphB4 (7). Therefore, although up-regulation of this receptor is part of the transcriptional reprogramming observed in intestinal adenomas triggered by Wnt activation, disruption of EphB4 signaling is important for tumor progression beyond this stage.

We have reported previously that EphB4 expression is reduced or lost in primary human colorectal tumors compared with the normal proliferating cells in intestinal crypts (7). Moreover, EphB4 levels are further reduced in lymph node metastases compared with primary tumors and there is frequent EphB4 promoter hypermethylation in colorectal tumors (7). In addition, we observed that low EphB4 tumor levels are significantly associated with shorter survival of colorectal cancer patients (7). Here, we report that targeted inactivation of a single allele of EphB4 in Apcmin/+ mice leads to a 25% shortening of animal survival and this is associated with increased proliferation and larger tumor size in the small intestine and a 9.6-fold increase in the number of tumors in the large intestine. Also, we show that reduced EphB4 expression results in changes in the expression of multiple genes involved in extracellular matrix remodeling, strongly suggesting that tumor cells expressing low EphB4 levels have increased migration/invasion potential. We directly tested this hypothesis and showed that low EphB4 levels are associated with increased invasion of colon cancer cells through a complex extracellular matrix. Collectively, in this study, we provide mechanistic insight showing that the loss of EphB4 results in increased proliferation and migration/invasion potential, contributing to the progression from small to large intestinal adenomas. In conclusion, our findings support the idea that EphB4 has tumor suppressor activities in intestinal tumorigenesis.

Disclosure of Potential Conflicts of Interest


Acknowledgments

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