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A Role for the Epidermal Growth Factor Receptor Signaling in Development of Intestinal Serrated Polyps in Mice and Humans

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Abstract

Background & Aims—Epithelial cancers can be initiated by activating mutations in components of the mitogen-activated protein kinase (MAPK) signaling pathway such as BRAF, KRAS, or epidermal growth factor receptor (EGFR). Human intestinal serrated polyps are a heterogeneous group of benign lesions, but some progress to colorectal cancer. Tumors that arise from these polyps frequently contain activating mutations in BRAF or KRAS, but little is known about the role of EGFR activation in their development.

Methods—Polyp samples were obtained from adults during screening colonoscopies at Mount Sinai Hospital in New York. We measured levels of EGFR protein and phosphorylation in human serrated polyps by immunohistochemical and immunoblot analyses. We generated transgenic mice that express the ligand for EGFR, HB-EGF, in the intestine.

Results—EGFR and the extracellular-regulated kinases (ERK)1/2 were phosphorylated in serrated areas of human hyperplastic polyps (HPPs), sessile serrated adenomas, and traditional serrated adenomas. EGFR and ERK1/2 were phosphorylated in the absence of KRAS or BRAF activating mutations in a subset of HPP. Transgenic expression of the EGFR ligand HB-EGF in the intestines of mice promoted development of small cecal serrated polyps. Mice that expressed a combination of HB-EGF and US28 (a constitutively active, G-protein-coupled receptor that increases processing of HB-EGF from the membrane) rapidly developed large cecal serrated polyps. These polyps were similar to HPPs and had increased phosphorylation of EGFR and

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ERK1/2 within the serrated epithelium. Administration of pharmacologic inhibitors of EGFR or MAP kinase to these transgenic mice significantly reduced polyp development.

Conclusions—Activation of EGFR signaling in the intestine of mice promotes development of serrated polyps. EGFR signaling is also activated in human HPPs, sessile serrated adenomas, and traditional serrated adenomas.

Keywords

colon cancer; colorectal tumorigenesis; neoplasm

INTRODUCTION

The two major groups of colorectal epithelial polyps are adenomas and serrated polyps. Adenomas are considered to be a precursor of colorectal cancer through a multistep mechanism that is initiated by mutations in the *APC* gene, a major component of the Wnt pathway. Subsequent progression to adenocarcinoma is associated with mutations in components of the MAPK signaling pathway, notably in *KRAS*^{1, 2}.

Serrated polyps encompass a heterogeneous group of lesions with characteristic “saw-tooth” epithelial infoldings, at least a subset of which has malignant potential³. They can be subdivided into at least three major groups: hyperplastic polyps (HPP), sessile serrated adenomas/polyps (SSA/P), traditional serrated adenomas (TSA)⁴.

Activating mutations in components of the MAPK pathway have been associated with serrated lesions. Activating mutations in *BRAF*, most commonly V600E, are frequently found in SSA/P (78 – 90%) and TSA (33 – 62%)⁵, but are rare in typical HPP³. Expression of *Braf*^{V600E} in mouse intestinal epithelial cells (IECs) leads to the development of hyperplastic crypts⁶.

KRAS mutations, most commonly G12D, are found in TSA (24 – 27%)⁵ and typical HPP (37%)³. In contrast, only 3 – 8% of the SSA/P have *KRAS* mutations⁵. In mice, expression of activated *KRAS* in IECs promotes the development of a hyperplastic epithelium with serrated features^{7–9}. *KRAS* and *BRAF* are thought to be part of the same MAPK signaling cascade and activating mutations in these genes are mutually exclusive³. Such mutations are thought to induce senescence through upregulation of p16^{INK4a}. Deletion of *Ink4a/Arf*, the p16^{INK4a} locus, promotes tumor progression^{6–9}.

One of the upstream components of the MAPK signaling pathway is the epidermal growth factor receptor (EGFR). EGFR is a transmembrane receptor tyrosine kinase of the ERBB (also known as HER) family of proteins that plays an important role in cellular proliferation and differentiation. Several reports link activation of EGFR to development of cancer. For instance, increased EGFR activity is observed in a subset of non–small-cell lung cancers¹⁰. For various cancers, aberrant EGFR expression or activity has been identified as an important factor^{11–13}. In the gastrointestinal tract, expression of EGFR and its ligands is often higher in tumors than in surrounding normal tissue¹⁴. Patients with colorectal cancer benefit from the treatment with EGFR specific antibodies unless their tumors contain activating mutations in either *BRAF* or *KRAS*¹⁵.

Here we report that EGFR is phosphorylated in human serrated polyps and that expression of an EGFR ligand, HB-EGF, in the intestine of transgenic mice leads to development of serrated polyps. Co-expression of HB-EGF with a constitutively active GPCR leads to increased processing of HB-EGF from the plasma membrane and to increased frequency and size of the polyps. In these polyps, we detected increased expression of *EGFR* and

phosphorylation of EGFR and ERK1/2, similar to what we observed in human serrated polyps. Collectively, these findings suggest a role for EGFR activation in the pathogenesis of serrated lesions.

METHODS

Mice

VS28 mice were described in Bongers et al.¹⁶. To generate the *HBGF* mice we cloned sequences encoding simian HB-EGF fused to GFP¹⁷ into a vector containing the mouse villin promoter¹⁸. The plasmid was verified by sequencing, and the transgene free of vector sequences was microinjected into C57BL/6J mouse eggs (The Jackson Laboratory). The resulting founders and their progeny (*HBGF* mice) were genotyped by PCR amplification of tail DNA. *HBUS* mice were generated by crossing *VS28* and *HBGF* mice. All experiments involving mice were performed in accordance with the guidelines of the Animal Care and Use Committee of Mount Sinai School of Medicine.

EGFR and MEK inhibitor experiments

The MEK inhibitor PD0352901 (12 mg/kg) and the EGFR inhibitor gefitinib (75 mg/kg) were dissolved in 0.2% (v/w) polysorbate-80 (Sigma) and administered daily by oral gavage for the time described in the text. PD0352901 was obtained from Mercachem (Nijmegen, The Netherlands) and gefitinib was synthesized at Mount Sinai by Dr. P. Reddy's group.

Human material

Four-micron sections were prepared from anonymized archival formalin-fixed paraffin embedded polyps obtained from adults undergoing routine screening colonoscopy at Mount Sinai Hospital. Sample acquisition followed Institutional Review Board guidelines.

Histology and immunofluorescence

Sections of paraffin-embedded tissues were subjected to immunofluorescent staining as described¹⁶. See Supplementary Material and Methods for details.

Isolation of intestinal epithelial cells (IECs)

Isolation of IECs was performed as described¹⁶. See Supplementary Material and Methods for details.

IEC proliferation

BrdU incorporation and FACS analysis of IECs was performed as described¹⁶. See Supplementary Material and Methods for details.

RNA extraction and quantitative PCR (qPCR)

RNA extraction and qPCR was performed as described¹⁶. See Supplementary Material and Methods for details.

Sequencing

DNA from mouse tissue or human paraffin sections were isolated, PCR amplified, purified and sequenced. See Supplementary Material and Methods for details.

Western blot

Western blot analysis was performed as described¹⁶. See Supplementary Material and Methods for details.

ELISA

The cDNAs containing US28¹⁶ and HB-EGF/GFP¹⁷ were subcloned into pcDEF3¹⁹ and transfected into Caco-2 cells using Lipofectamine. Twenty-four hours after transfection the supernatants were used for a HB-EGF ELISA (Cat# DY259, R&D systems). See Supplementary Material and Methods for details.

Statistical analysis

Statistical analyses analysis was performed as described¹⁶. $P < 0.05$ was considered significant. All results shown represent mean $\pm$ s.e.m. See Supplementary Material and Methods for details.

RESULTS

Phosphorylation of the EGFR receptor in human serrated polyps

To investigate EGFR signaling in serrated polyps, we examined the phosphorylation of EGFR in tissue sections of 27 hyperplastic polyps (HPP), 25 sessile serrated adenomas (SSA/P), 22 traditional serrated adenomas (TSA), and 14 normal controls, using an antibody that specifically recognizes the autophosphorylated Y1173 residue of EGFR. We found P-EGFR/Y1173 positive staining in 70% of HPP, 48% of the SSA/P and 91% of the TSA cases examined. P-EGFR/Y1173 staining was found in less than 1% of the epithelial cells of normal controls. In marked contrast, a significant proportion of cells were positive within the serrated lesions (~33% in HPP, ~23% in SSA/P and ~31% in TSA cases) (Figure 1A – B, 6E – F, Supplemental Table I).

Expression of EGFR ligands is often higher in human gastrointestinal tumors than in surrounding normal tissue¹⁴. Next, we evaluated expression of one of the EGFR ligands, HB-EGF, in the same set of human polyps using an anti-HB-EGF antibody. HB-EGF expression was detected in 4% of HPP, 4% of the SSA/P, and 9% of the TSA cases (Figure 1C – D, Supplemental Table I). Among the positive cases, HB-EGF staining was detected in 50, 50, and 65% of the epithelial cells of HPP, SSA/P, and TSA respectively. No HB-EGF staining was detected in the epithelium of normal controls.

Several cell surface receptors, including EGFR, signal through the MAPK pathway. We examined next the phosphorylation of ERK1/2 in normal intestinal tissue and in human serrated polyps. We detected positive staining for phospho-ERK1/2 (P-ERK1/2) in 36% of the normal biopsies (Figure 1E – F, Supplemental Table I). In these normal cases, 5 – 10% of the epithelial cells were positive. In striking contrast, we found P-ERK1/2 staining in all HPP, SSA/P, and TSA cases examined. P-ERK1/2 staining was observed in 10 – 80% of the serrated epithelial cells. In the normal epithelium P-ERK1/2 staining was found predominantly in the cytosol, whereas in the HPP, SSA/P, and TSA cases it was also frequently found in the nucleus.

Human HPP, SSA/P, and TSA cases frequently harbor mutations in either *KRAS* or *BRAF*⁵ and this could account for increased ERK1/2 activation. To examine if P-ERK1/2 positivity correlated with the presence of known mutations, we analyzed all cases for mutations in *BRAF*⁶⁰⁰ and *KRAS*^{12/13} (Supplemental Table 1). *BRAF* or *KRAS* mutations were not found in the normal cases. *BRAF* mutations were found in 22% of the HPP, 88% of the SSA/P and 68% of the TSA cases. *KRAS* mutations were found in 30% of HPP, 8% of SSA/P and 23% of TSA cases. Interestingly, 62% of the HPP cases that were WT for both *KRAS* and *BRAF*, were positive for P-EGFR (Supplemental Table 1).

In summary, our results indicate that: 1) EGFR is phosphorylated in human serrated polyps, 2) HB-EGF is expressed within a small subset of serrated lesions, 3) MAPK activation is found in all cases examined, and 4) a subset of HPP, not bearing *KRAS* and *BRAF* mutations, show phosphorylated EGFR.

Increased number of IECs in *HBGF* mice

The results described above suggested that EGFR activation could be important in the pathogenesis of serrated polyps. To test this hypothesis we created transgenic mice that expressed the EGFR ligand HB-EGF (*HBGF* mice) in the intestine. HB-EGF is a transmembrane protein that is expressed by rare cells in the lamina propria²⁰, but not by intestinal epithelial cells (Supplemental Figure 1A). HB-EGF can signal in a juxtacrine, autocrine and/or paracrine manner. The mode of action of HB-EGF is determined by the degree of proteolytic release of the ligand from the cell membrane²¹.

DNA sequences encoding a HB-EGF and GFP C-terminal fusion protein (HB-EGF/GFP) were placed under the control of the villin promoter, which targets transgene expression predominately to the IECs of both the small and large intestine¹⁸. Two transgenic lines (8 and 32) were established from four founders and the resulting mice were healthy and fertile. To examine distribution of HB-EGF in the intestine, we performed immunostaining with a HB-EGF antibody. HB-EGF expression was detected in IECs of transgenic but not WT mice (Figure 2, A and B, Supplemental Figure 1B). The staining was observed in cells in the villi and crypts throughout the intestine, indicating that *HBGF* mice expressed HB-EGF in the intestinal epithelium.

Analysis of *HBGF* mice showed no major abnormalities, except for an increased diameter of the gut and an increased number of IECs (Figure 2C). Histological examination of the tissue after EDTA treatment showed that the increased number of IECs was not due to differences in cell attachment (Supplemental Figure 2A). Morphometric analysis of histological sections showed that the increased number of IECs was not due to increased length of the crypt and villi (Supplemental Figure 2B). To examine whether the increased cellularity and increased gut size were caused by increased cellular proliferation, we determined BrdU incorporation in epithelial cells by flow cytometry. The proportion of cells incorporating BrdU did not differ between samples obtained from *HBGF* and WT littermates, indicating that the changes observed between the groups were not due to changes in cell proliferation (Figure 2C).

To examine if the increased number of IECs in *HBGF* mice could be explained by increased senescence (as proposed for mice expressing *Kras*^{G12D} in IECs⁸), we determined the expression of the senescence marker *p16*^{INK4a} by qPCR. *HBGF* mice had increased levels of *p16*^{INK4a} mRNA in IECs from the small intestine (Figure 2C). These results suggest that expression of HB-EGF in the intestine promoted an increase in the number of IECs by inducing senescence rather than cell proliferation.

Development of small cecal polyps with serrated features in *HBGF* mice

The intestine of the *HBGF* mice was histologically unremarkable when examined at 10 weeks of age. However, by 16 weeks of age, small polyps with a diameter of approximately 2 – 3 mm (Figure 2D) were observed in approximately 8% of the mice in line 8 (*n* = 25) and in 4% of mice in line 32 (*n* = 24). Histologically, the polyps consisted of crypts with narrow, tapered bases and expanded, serrated upper regions that occupied 50 – 90% of the crypt axis (Figure 2E). The basal epithelium was composed of mitotic cells and an expanded symmetrical proliferative zone. The upper serrated epithelium consisted of cytologically bland columnar cells with pale eosinophilic cytoplasm and normochromatic nuclei. Mature

mucin-producing goblet cells were evenly distributed. These results indicate that expression of HB-EGF in the intestine leads to the development of serrated polyps in mice.

Increased cleavage of HB-EGF by co-expression of a constitutively active GPCR

We hypothesized that the development of serrated polyps in *HBGF* mice was caused by activation of the EGFR by cleaved HB-EGF. G_q and G_i -coupled GPCRs are known to promote ectodomain shedding in a metalloprotease^{22, 23} or Src-dependent manner²⁴.

To test if HB-EGF could be cleaved in vitro by a GPCR, we transiently transfected intestinal epithelial (Caco-2) cells with HB-EGF/GFP and US28, a constitutively active GPCR that can activate G_q and G_i proteins²⁵. To determine if US28 promoted cleavage of HB-EGF, we measured HB-EGF by ELISA in the supernatants 24 hours after transfection. We found that the samples co-transfected with US28 had higher levels of soluble HB-EGF in the supernatants than the samples transfected with HB-EGF alone (Supplemental Figure 3).

Expression of the constitutively active GPCR US28 exacerbates development of serrated polyps induced by HB-EGF

To test if US28 expression could affect the phenotype elicited by HB-EGF in vivo, we crossed *HBGF* mice (line 8) with mice expressing US28 in IECs (*VS28* mice, line 18)¹⁶.

US28 and HB-EGF double transgenic mice (referred to as *HBUS* mice) developed an early and striking phenotype compared to the *HBGF* mice. Starting at 5 weeks of age, *HBUS* mice ($n = 85$) spontaneously developed macroscopic polyps in the cecum with a diameter of 2 – 13 mm (Figure 3B). After 16 weeks of age, the majority (86%) of *HBUS* mice examined had polyps (Figure 3A). No polyps were observed in the cecum of WT ($n = 25$) or *VS28* mice ($n = 24$, 16 – 82 weeks).

The polyps in *HBUS* mice were protuberant, sessile, and well-circumscribed. Microscopically, they were similar to the polyps observed in the *HBGF* mice and consisted of crowded, parallel, vertically symmetrical crypts with narrow, tapered bases and expanded, serrated upper regions that occupied 50 – 90% of the crypt axis (Figure 3, C and D). The basal epithelium was proliferative and mitotically active whereas the upper crypts were lined by cytologically bland columnar epithelial cells with pale eosinophilic cytoplasm, normochromatic nuclei and low nuclear-cytoplasmic ratios interspersed with mature goblet cells that produced basophilic mucin.

Similar to hyperplastic polyps in humans (Figure 3E), the murine lesions had straight crypts with tubular crypt bases, symmetrical proliferative zones, surface epithelial maturation, absence of cytological atypia and evenly distributed goblet cells. Over time, microscopic evaluation revealed the development of dysplastic lesions within up to 25% of the polyps (Figure 3, F). The focal dysplasia was characterized by patchy replacement of the mature serrated epithelium by circumscribed groups of poorly differentiated, mitotically active columnar epithelium with crowded, hyperchromatic nuclei and high nuclear-cytoplasmic ratios (Figure 3F). Polyps that contained dysplastic foci also had corresponding foci of Ki67 expression outside the basal crypts. Taken together these results indicate that the expression of the GPCR US28 by intestinal cells significantly increased the incidence of the serrated polyps induced by HB-EGF (Figure 3A).

Serrated polyps in *HBUS* mice resemble human hyperplastic polyps

As described above, *HBUS* mice develop serrated polyps that histologically resemble human hyperplastic polyps. To further classify these lesions we examined the expression

pattern of the proliferation marker Ki67⁴ and of the DNA repair genes *Mlh1*, *Msh2*, *Msh6* and *Mgmt*.

Immunostaining showed that Ki67 positive cells were found only in the crypt base of the cecum of WT mice (Figure 4A). An elongated, but symmetric basal-dominant pattern of Ki67 (Figure 4B), similar to that seen in human hyperplastic polyps⁴, was found in serrated polyps of *HBUS* mice ($n = 11$).

Expression of repair genes is altered in serrated lesions. MGMT expression is decreased in HPP, SSA/P, and TSA. Expression of MLH1 is decreased in SSA/P, but not in HPP or TSA³. To examine repair gene expression in *HBUS* polyps, we isolated mRNA from polyps and normal surrounding tissue. We found that *HBUS* polyps had decreased mRNA expression of *Mgmt* but not of *Mlh1*, *Msh2*, and *Msh6* (Figure 4C). Thus, based on histopathological parameters, Ki67 expression pattern, and repair gene expression, we conclude that *HBUS* polyps most closely resemble human hyperplastic polyps.

Increased expression and phosphorylation of the EGFR receptor in serrated polyps of *HBUS* mice

HBGF and *HBUS* mice express HB-EGF throughout the gut but, remarkably, serrated polyps were only observed in the cecum. EGFR is abnormally activated in many epithelial tumors and several mechanisms lead to aberrant receptor activation, including receptor overexpression and gene amplification²⁶. To test if aberrant expression of EGFR could underlie the strict cecal localization of the polyps, we extracted RNA from IECs isolated from polyps and surrounding tissue and performed qPCR. We found that expression of *Egfr* mRNA was elevated in the polyps compared to normal surrounding tissues, or WT cecum (Figure 5A, Supplemental Figure 4). HB-EGF acts both on EGFR and on ERBB4²⁷. To check if the effects of HB-EGF could be partially mediated through ERBB4, we determined its expression through qPCR. *ErbB4* mRNA expression was detected in WT heart, but not in IECs isolated from WT ceca, *HBUS* polyps, or from tissue surrounding polyps (Figure 5A). These results suggested that EGFR is the main receptor mediating effects induced by HB-EGF in the cecum of *HBUS* mice.

Next, we evaluated the levels of EGFR protein and its phosphorylation by Western blot analysis. Consistent with the mRNA results, the levels of EGFR protein were increased in the polyps of *HBUS* mice, compared to surrounding normal cecum, or cecum from WT mice (Figure 5B). EGFR ligand binding induces receptor dimerization and subsequent autophosphorylation of tyrosine residues (e.g. Y1068 or Y1173).. To investigate if EGFR was activated we used an antibody that specifically recognizes. P-EGFR/Y1068. EGFR phosphorylation was increased in the polyps of *HBUS* mice compared to normal surrounding tissue or tissue from WT mice (Figure 5B). To determine if these changes took place in the intestinal epithelium we performed immunostaining. We found positive P-EGFR/Y1173 staining in all *HBUS* polyps examined ($n = 13$). In all cases, P-EGFR/Y1173 staining was confined to the serrated epithelium (Figure 5D), similar to what we observed in human lesions (Figure 1B). No P-EGFR/Y1173 staining was detected in tissues surrounding *HBUS* polyps, nor in ceca of WT mice ($n = 5$).

These results suggest that activation of the EGFR receptor plays a role in the development of intestinal serrated lesions in mice.

Increased ERK1/2 phosphorylation in serrated polyps of *HBUS* mice

EGFR signaling is known to activate the MAPK pathway leading to increased phosphorylation of P-ERK1/2. To test whether *HBUS* mice had activation of ERK1/2, we determined the levels of Thr²⁰²/Tyr²⁰⁴ phosphorylation of ERK1/2 (P-ERK1/2) in cecal

extracts by Western blotting. P-ERK1/2 phosphorylation was increased in the polyps of *HBUS* mice compared to normal surrounding tissue or tissue from WT mice (Figure 5C).

Activating mutations in either *BRAF* or *KRAS* are associated with serrated polyps in humans^{3,5}. To rule out that *HBUS* mice had mutations in *Braf*^{V600} or *Kras*^{G12}, we sequenced DNA isolated from 16 polyps of *HBUS* mice. We found no evidence for mutation of the *Braf*^{V600} or *Kras*^{G12} residues in the 16 samples tested.

The intestinal epithelium is constantly renewed from crypt stem-cells that give rise to the partially differentiated transit amplifying cells and eventually to the post-mitotic fully differentiated cells²⁸. To localize the site of ERK1/2 phosphorylation we performed immunostaining on cecal samples isolated from different mice. We found that ERK1/2 phosphorylation was predominantly localized to basal IECs in approximately 40% of the crypts in the ceca of WT mice (data not shown). In contrast, phosphorylated ERK1/2 was absent in the basal crypts, but present in the serrated IECs of *HBUS* polyps ($n = 22$; Figure 5E).

EGFR signaling can activate the MAPK pathway leading to increased phosphorylation of P-ERK1/2. To determine if P-EGFR and P-ERK1/2 were found in the same cells, we stained serrated polyps with P-EGFR and P-ERK1/2 (MAPK-YT) antibodies. We found that P-EGFR was detected in cells that were also positive for P-ERK1/2 in both murine (Figure 5F) and human serrated polyps (Supplemental Figure 5).

These combined data indicate that polyps of *HBUS* mice have a dysregulated ERK1/2 signaling, similar to what was observed in human serrated polyps (Figure 1D).

Prevention of polyp formation in *HBUS* mice by pharmacological inhibition of EGFR and MEK

We showed above that phosphorylation of EGFR and ERK1/2 is affected in serrated polyps of *HBUS* mice, which suggests that they may be mechanistically important for development of polyps. The cleavage of the membrane-anchored form of HB-EGF produces a soluble form of the growth factor and a remnant C-terminal peptide. Soluble HB-EGF binds EGFR, ERBB4, NRDC, and heparan sulfate proteoglycan, whereas the C-terminal peptide signals in a non-receptor mediated manner^{29,30}. To test if signaling of HB-EGF in the *HBUS* mice is mediated through the EGFR we treated *HBUS* mice with gefitinib²⁷. Gefitinib is a submicromolar small-molecule specific EGFR inhibitor that competes reversibly with ATP for binding to the intracellular catalytic domain and thereby inhibits EGFR autophosphorylation and downstream signaling^{26,27}.

EGFR can activate several signaling pathways^{26,31}. To test if the EGFR-mediated activation of the MAPK pathway is important for the formation of serrated polyps, we used the specific MEK1/2 inhibitor PD0325901. PD0325901 is an orally active non-ATP-competitive specific inhibitor of MEK1/2 with nanomolar affinity in vitro³².

Adult *HBUS* mice were randomly assigned to be treated p.o. for 9 weeks with 75 mg/kg gefitinib, 12.5 mg/kg PD0325901, or vehicle (Figure 6A). As expected, 64% of the vehicle treated *HBUS* mice developed polyps with a size of 2.3 ± 0.5 mm ($n = 25$). Treatment with the EGFR inhibitor gefitinib significantly reduced the incidence (7.1%; $P = 0.0007$, $n = 14$) as well as the size of the polyps (0.03 ± 0.03 mm; $P < 0.01$). The only gefitinib treated *HBUS* mouse that developed a polyp showed a marked decrease in the number of P-ERK1/2 positive cells in the serrated crypts compared to vehicle treated *HBUS* mice (Supplemental Figure 6).

PD0325901-treated *HBUS* mice showed a significant reduction in the polyp incidence (25%; $P = 0.039$; $n = 12$) and polyp size compared to vehicle treated mice (0.58 ± 0.39 mm; $P < 0.05$; Figure 6, B and C).

These results indicate that both the MAPK pathway and EGFR signaling are important for the development of serrated polyps in *HBUS* mice.

DISCUSSION

In this report, we provide evidence that activation of EGFR signaling is important for development of intestinal serrated polyps. We show that EGFR is selectively activated (phosphorylated) in human and murine polyps, and that expression of the EGFR ligand HB-EGF in intestinal epithelial cells of transgenic mice leads to development of serrated polyps that are remarkably similar to those found in humans. To our knowledge, this is the first report indicating that increased EGFR signaling promotes the formation of intestinal serrated polyps.

Increased expression of EGFR or its ligands is associated with various types of cancer^{11–13}. In colorectal cancer, expression of EGFR and its ligands is often higher in tumors compared to normal surrounding tissue¹⁴ and a subset of patients, that are wild-type for KRAS and BRAF, benefit from EGFR-antibody treatment¹⁵. Here we report that 70% of HPP, 48% of the SSA/P and 91% of the TSA cases have an increased number of P-EGFR positive epithelial cells compared to normal tissue. Furthermore, we found expression the EGFR ligand HB-EGF in a small subset of patients (4% of the HPP and SSA/P cases, and in 9% of the TSA cases).

Based on these findings, we decided to test whether HB-EGF expression in the intestine could promote development of serrated polyps in mice. Our animals, similar to those generated by Chen et al., had increased numbers of IECs³³. The increased number of cells did not lead to increased susceptibility to cancer development, but was associated with the development of small serrated polyps after 16 weeks of age. These polyps were found in less than 10% of the mice in two independent transgenic lines. The incidence of such polyps was greatly increased by co-expression of HB-EGF with US28, a constitutively active GPCR that promotes shedding of HB-EGF from the membrane, as shown here. This finding prompted us to investigate the expression of US28 in human serrated lesions. Thus far, we have not been able to detect US28 expression in human serrated polyps ($n = 10$, data not shown). However, because of the small sample analyzed, we cannot categorically dismiss at this point that US28 or the virus encoding it (human cytomegalovirus) have a role in pathogenesis of serrated lesions in humans.

As described above, *HBUS* polyps are most similar to human HPP based on histopathological parameters, Ki67 expression pattern, and repair gene expression. Notably, they lack the protuberant growth pattern with viliform projections, stratified enlarged pencil-shaped hyperchromatic nuclei observed in TSA, and the significant crypt branching or basal crypt dilation typical of human SSA/P. However, different from human HPP, the polyps of *HBUS* mice are relatively large and are found in the cecum, whereas human HPP are predominantly found in the left colon.

EGFR signaling as well as activating mutations in *BRAF* and *KRAS* can activate the MAPK pathway³⁴. *BRAF* and *KRAS* activating mutations are associated with development of serrated lesions in humans^{3, 5}. Interestingly, we found increased activation of the MAPK pathway in HPP cases that were wild-type for both *BRAF*^{V600} and *KRAS*^{G12}. Among these wild-type HPP cases, 62% showed evidence for EGFR activation, suggesting that in the absence of activating mutations in KRAS or BRAF; aberrant EGFR activity might elicit

activation of the MAPK pathway. Therefore, we propose that, similar to activating mutations in *BRAF* or *KRAS*, increased EGFR activity may promote the development of serrated polyps in humans. Consistent with this view is the observation that activation of EGFR by expression of HB-EGF in transgenic mice leads to development of serrated lesions that resemble human hyperplastic polyps.

In mice, ERK1/2 phosphorylation is normally present in the crypt base of cecal IECs, whereas in murine serrated polyps it was detected predominantly in the serrated epithelium. This altered P-ERK1/2 localization might indicate a change in cellular differentiation in the serrated polyps. Indeed MAPK signaling has been shown to be important for IEC differentiation³⁵. The importance of the MAPK pathway in the development of serrated polyps in *HBUS* mice is further highlighted by the experiments using the MEK1/2 inhibitor PD0325901. MEK inhibition significantly reduced the incidence as well as the size of the serrated polyps,

The metalloprotease-dependent cleavage product of HB-EGF resembles EGF, and is a potent EGFR and ERBB4 ligand²⁹. In *HBUS* mice, only EGFR is expressed in the *HBUS* polyps, suggesting that it may directly mediate HB-EGF function. Indeed, treatment of the *HBUS* mice with the EGFR inhibitor gefitinib causes a marked reduction in the number and size of polyps. These results strongly suggest that EGFR activation by HB-EGF is critical for disease development in *HBUS* mice, but they do not rule out that activation of other tyrosine kinase receptors may also be important for pathogenesis, because gefitinib may also block other tyrosine kinase receptors^{36, 37}.

The mechanism whereby increased MAPK leads to development of serrated lesions is unclear. Increased senescence could underlie the development of such lesions. Indeed, the *HBGF* mice described here had increased expression of the cell cycle regulator and senescence marker *p16^{INK4a}*, which could explain the increased number of IEC. Accordingly, activating mutations in either *Kras^{G12D}* or *Braf^{V600E}*, which also lead to activation of the MAPK pathway, also induce expression of *p16^{INK4a}* in IECs and promote the development of serrated colonic crypts^{6, 8}. However, the relevance of senescence to development of serrated lesions has been questioned by Feng et al., who have not been able to detect changes in the levels of *p16^{INK4a}* in the serrated lesions of mice or humans⁷. While the role of *p16^{INK4a}* in the initial development of serrated polyps remains unclear, the available evidence suggests that its inactivation of *p16^{INK4a}* in the presence of *Kras^{G12D}* expression leads to development of invasive carcinomas^{6, 8}. It will thus be important to test if inactivation of *p16^{INK4a}* in *HBUS* mice will also promote development of carcinomas.

All polyps were found in the cecum of *HBUS* mice, despite expression of HB-EGF throughout the intestine. What could account for this striking topological distribution? We suggest that the cecal localization could be dependent on the site-specific activation of EGFR, e.g. through cecum-restricted expression of matrix metalloprotease(s) or Src proteins required for cleavage of membrane-tethered HB-EGF³⁸. Alternatively, a specific environmental factor, such as the local microbiota could synergize with the genetic changes described here to promote increased EGFR activation. Specific microbes could contribute directly to increased shedding of HB-EGF, as has been shown for e.g. *H. pylori*^{39, 40}, or indirectly by promoting infiltration of leukocytes that promote the release of EGFR ligands^{41–45}. The animal models described here should allow for the precise testing of these hypotheses.

In summary, our studies show that activation of EGFR is mechanistically important for development of serrated polyps in transgenic mice. These findings, coupled to the demonstration that EGFR is activated in human polyps that do not bear canonical mutations

in *BRAF* or *KRAS*, suggest that it may have a role in the development of serrated lesions in humans. Whether HB-EGF, another EGFR ligand, or EGFR activating mutations are important for development of human polyps remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

IECs	intestinal epithelial cells
NCS	Newborn Calf Serum
SSA/P	sessile serrated adenoma/polyps
TSA	traditional serrated adenomas
HPP	hyperplastic polyps
EGFR	epidermal growth factor receptor

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Figure 1. Increased EGFR and MAPK phosphorylation in human serrated polyps compared to normal mucosa

(A) In the normal mucosa EGFR autophosphorylation of residue Y1173 (red, P-EGFR) is found predominantly in the lamina propria (arrow) and in occasionally (< 1%) in epithelial cells (*). (B) In human serrated polyps, P-EGFR is found in the serrated epithelium. Inset shows higher magnification of the boxed area. (C–D) A subset of serrated polyps (D) expressed HB-EGF (red), whereas the normal tissues (C) were negative. Inset shows higher magnification (D, boxed). (E) In normal mucosa P-ERK1/2 predominantly detected in inflammatory cells (arrow). Inset shows a higher magnification of the boxed area. (F) In serrated polyps, P-ERK1/2 immunoreactivity is seen in the serrated epithelium extending

towards the lumen, but is absent at the crypt base. P-ERK1/2 staining was superimposed over pan-keratin (Pan-Ker, green). For an overview see Supplemental Table I. Shown are normal mucosa (Normal, *A, C, E*), a human hyperplastic polyp (HPP, *B, D*) and a human sessile serrated adenomas (SSA/P, *F*). Sections were counterstained with DAPI (*A – F*, blue) and pan-keratin (*A, B, E, F*, Pan-Ker, green). Scale bars: 100 μm (*A – F*), 25 μm (Inset *B, D* and *E*).

Figure 2. Mice expressing HB-EGF in IECs develop serrated polyps

(A and B) Representative pictures of the jejunum of WT (A) and HBGF (B) mice stained with HB-EGF (red) and DAPI (blue). (C) Compared to WT mice, the small intestine of HBGF mice had an increased intestinal diameter ($n = 5 - 9$), an increased number of IECs ($n = 9 - 16$), an equivalent number of proliferating IECs as determined by BrdU incorporation ($n = 3 - 5$), and an increase mRNA expression of the senescence marker $p16^{\text{INK4a}}$ ($n = 3 - 4$). (D) A polyp in the cecum of a HBGF mouse (marked by dashed line). (E) H&E stained section of a polyp from a HBGF mouse showing the presence of serrated crypts. Inset shows a higher magnification of the dashed area. NS not significant, * $P < 0.05$, *** $P < 0.001$ compared to WT. Scale bar: 100 μm (A – B, E and inset E).

Figure 3. Expression of US28 exacerbates development of polyps induced by HB-EGF
 (A) Increased incidence of serrated polyps (SP) in *HBUS* mice ($n = 85$) compared to *HBGF* mice ($n = 34$). (B) Polyp in the cecum of a *HBUS* mouse. (C–E) H&E stained sections of ceca from *HBUS* mice (C–D) and a human serrated polyp (E) showing the presence of serrated crypts. Arrows indicate representative serrated crypts (D–E). (F) Focal of dysplasia in a serrated polyp from a *HBUS* mouse (arrows), inset shows a higher magnification of a dysplastic crypt *** $P < 0.001$, scale bars: 100 μm (B–E).

Figure 4. Pattern of cellular proliferation and expression of repair genes in *HBUS* polyps (*A – B*) Compared to WT (*A*), serrated polyps from *HBUS* mice (*B*) have elongated, but symmetric basal-dominant pattern of Ki67 (red). Sections were counterstained with pan-keratin (Pan-Ker, green) and DAPI (blue). Bars to the right of each panel indicate length of the proliferative zone (red). (*C*) Decreased expression of *Mgmt* mRNA, but not of *Mlh1*, *Msh2*, *Msh6* in tissue isolated from *HBUS* polyps compared to normal surrounding tissue ($n = 5$, $*P < 0.05$). Scale bars: 100 μ m (*A – B*).

Figure 5. Modulation of EGFR and ERK1/2 signaling in serrated polyps of *HBUS* mice
 (A) Relative expression (Rel. Exp.) of *Egfr* and *ErbB4* mRNA in IECs isolated from serrated polyps of *HBUS* mice compared to WT and normal (Norm.) surrounding tissue ($n = 6$, for each). WT heart tissue was used as a positive control for ERBB4. (B) Western analysis of phosphorylated EGFR (P-EGFR), total EGFR and β -actin in the ceca of WT or *HBUS* mice. Densitometric analysis shows that relative immunoreactivity (RI) of P-EGFR is significantly increased in the polyps of *HBUS* mice compared to WT and normal surrounding cecal tissue (Norm.) ($n = 3$). (C) Western analysis of phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK1/2) in the ceca of WT or *HBUS* mice. Densitometric analysis shows that relative immunoreactivity (RI) of P-ERK1/2 is significantly increased in the polyps of *HBUS* mice compared to WT and normal surrounding cecal tissue (Norm.) ($n = 8 - 12$). (D) Phosphorylated EGFR (red, P-EGFR) in serrated polyps found in *HBUS* mice is confined to the serrated epithelium. (E) In serrated polyps from *HBUS* mice P-ERK1/2 immunoreactivity (red) is seen in the serrated epithelium extending towards the lumen, but is absent at the crypt base ($n = 14$). To enhance visualization, P-ERK1/2 staining was superimposed over DAPI (blue) and pan-keratin (Pan-Ker, green). (F) Phosphorylated ERK1/2 (green, MAPK-YT) is found in the same cells as P-EGFR (red) in polyps of *HBUS* mice ($n = 3$). Sections were counterstained with DAPI (blue) and pan-keratin (B – D, E). ** $P < 0.01$, *** $P < 0.001$ compared to WT and normal surrounding tissue. Scale bars: 250 μ m (B, D), 100 μ m (E, F)

Figure 6. Reduced incidence of serrated polyps in *HBUS* mice treated with the MEK inhibitor PD0325901 (PD) and the EGFR inhibitor gefitinib (Gef)

(A) At 7 weeks of age *HBUS* littermates were randomly assigned to either receive daily p.o. vehicle (Veh, 1% polysorbate-80, $n = 25$), gefitinib (75 mg/kg, $n = 14$) or PD0325901 (12.5 mg/kg, $n = 12$) for 9 weeks. (B – C) At 16 weeks of age, mice were euthanized and analyzed for the presence of polyps. Treatment with gefitinib ($P = 0.0007$) or PD0325901 ($P = 0.038$) significantly reduced the incidence of polyps (B). Size of the serrated lesions was determined by histological measurement. Gefitinib and PD0325901 treated *HBUS* mice had significantly smaller polyps compared to vehicle treated *HBUS* mice (C). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to vehicle.