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Antitumor activity of the MEK inhibitor trametinib on intestinal polyp formation in Apc\textsuperscript{Δ716} mice involves stromal COX-2

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Key words
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Cryptic signal-regulated kinase is an MAPK that is most closely associated with cell proliferation, and the MEK/ERK signaling pathway is implicated in various human cancers. Although epidermal growth factor receptor, KRAS, and BRAF are considered major targets for colon cancer treatment, the precise roles of the MEK/ERK pathway, one of their major downstream effectors, during colon cancer development remain to be determined. Using Apc\textsuperscript{Δ716} mice, a mouse model of familial adenomatous polyposis and early-stage sporadic colon cancer formation, we show that MEK/ERK signaling is activated not only in adenoma epithelial cells, but also in tumor stromal cells including fibroblasts and vascular endothelial cells. Eight-week treatment of Apc\textsuperscript{Δ716} mice with trametinib, a small-molecule MEK inhibitor, significantly reduced the number of polyps in the large size class, accompanied by reduced angiogenesis and tumor cell proliferation. Trametinib treatment reduced the COX-2 level in Apc\textsuperscript{Δ716} tumors in vivo and in primary culture of intestinal fibroblasts in vitro. Antibody array analysis revealed that trametinib and the COX-2 inhibitor rofecoxib both reduced the level of CCL2, a chemokine known to be essential for the growth of Apc mutant polyps, in intestinal fibroblasts in vitro. Consistently, trametinib treatment reduced the Ccl2 mRNA level in Apc\textsuperscript{Δ716} tumors in vivo. These results suggest that MEK/ERK signaling plays key roles in intestinal adenoma formation in Apc\textsuperscript{Δ716} mice, at least in part, through COX-2 induction in tumor stromal cells.

Colorectal cancer is one of the leading causes of cancer-related deaths, and its progression is considered to involve various genetic and epigenetic changes.

Heterozygous Apc mutant mice develop adenomatous polyps in the intestines, and are widely used as a genetically-engineered model of familial adenomatous polyposis and early-stage sporadic colon cancer.\(^1\)\(^-\)\(^7\) Loss of heterozygosity at Apc loci in intestinal epithelial cells of the Apc mutant mice leads to Wnt signaling activation, resulting in tumor initiation in the intestines.\(^2\)\(^-\)\(^4\) However, activation of Wnt signaling is not sufficient for tumor formation; we and others reported earlier that the growth of intestinal tumors in Apc mutant mice required induction of COX-2 in tumor stromal cells and activation of mammalian target of rapamycin complex 1 (mTORC1) in tumor epithelial cells.\(^3\)\(^-\)\(^7\) We subsequently found that JNK activation in adenoma epithelial cells of Apc mutants was responsible for mTORC1 activation and hence tumor formation.\(^8\)

The MAPK family consists of ERK, JNK, and p38 MAPK, and ERK is most closely implicated in the regulation of cell proliferation and survival, as well as in oncogenic transformation.\(^9\)\(^,\)^\(^10\) In colorectal cancer, epidermal growth factor receptor, KRAS, and BRAF are considered major targets for therapy, and MEK/ERK is one of their important downstream effectors. However, the roles of the MEK/ERK signaling in the formation of intestinal adenomas prior to the acquisition of KRAS or BRAF mutations are not fully understood.

Trametinib is an allosteric inhibitor of MEK1/2,\(^11\)\(^,\)^\(^12\) and has been approved by the FDA for treating patients with metastatic melanoma harboring the BRAF\(^{V600E/K}\) mutation.\(^9\)\(^,\)^\(^10\) Here we show that treatment with trametinib suppresses the growth of intestinal polyps in Apc\textsuperscript{Δ716} mice. Activation of ERK was observed not only in a subset of adenoma epithelial cells, but also in stromal cells including fibroblasts and vascular endothelial cells, and trametinib treatment reduced the levels of COX-2 in tumors in vivo and intestinal fibroblasts in vitro, suggesting a role for stromal MEK/ERK signaling in intestinal tumor formation.

Materials and Methods

Animals and drug treatments. The construction of Apc\textsuperscript{Δ716} mice was described previously.\(^2\) Male and female Apc\textsuperscript{Δ716} mice at 10 weeks of age were gavaged with trametinib (2 mg/kg/day; LC Laboratories, Woburn, MA, USA) or vehicle (2% acetic acid, 10% polyoxyethylene(10) castor oil, and 10% PEG400) for 8 weeks.\(^12\)

All animal experiments were carried out according to the protocols approved by the Animal Care and Use Committee of Aichi Cancer Center Research Institute (Nagoya, Japan).
Microvessel density. Microvessel density was determined as described previously.\(^{(5)}\)

**Western blot analysis.** Western blot analysis was carried out as described previously,\(^{(5)}\) using primary antibodies for \(\beta\)-actin (Sigma-Aldrich, St. Louis, MO), phosphorylated p-S6, p-Akt, p-ERK1/2, p-JNK, p-p38, p-STAT3, COX-1 (Cell Signaling Technology, Danvers, MA, USA), and COX-2 (Cayman Chemical, Ann Arbor, MI, USA).

**Angiogenesis antibody array analysis.** Primary mouse intestinal fibroblasts were serum-starved with 0.1\% FBS for 12 h.

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Fig. 1. Activation of ERK in intestines of WT and Apc\(^{716}\) mice. (a) Western blot analysis for ERK phosphorylation in various parts of the intestines of WT C57BL/6 mice treated with trametinib or vehicle (control). Col, colon; Duo, duodenum; Ile, ileum; Jej, jejunum. (b) Immunostaining of p-ERK in intestines of WT mouse treated with trametinib or vehicle (control) for 1 week. Lower panels of each section show higher magnification of the boxed areas in the corresponding upper panels. Bar = 50 \(\mu\)m. (c) Western blot analysis for effects of trametinib treatment on signaling pathways in the ileum of WT mouse. (d) Western blot analysis for effects of trametinib treatment on signaling pathways in normal intestinal mucosa (N) and polyps (P) of Apc\(^{716}\) mouse. (e) Immunostaining of p-ERK in intestinal polyps of Apc\(^{716}\) mouse treated with trametinib or vehicle (control) for 1 week. Lower panels show higher magnifications of the boxed areas in the upper panels. Bars, upper panels = 200 \(\mu\)m; lower panels = 50 \(\mu\)m.
then cultured in DMEM with 10% FBS in the presence oftrametinib (10 nM), rofecoxib (10 μM; Sigma), or vehicle control (DMSO) for 16 h. Preparation of whole cell lysates and antibody array analysis was carried out according to the manufacturer’s protocol (Mouse Angiogenesis Array Kit, ARY015; R&D Systems, Minneapolis, MN).

**Statistical analysis.** Statistical analyses were carried out using R (version 3.0.2; R Foundation for Statistical Computing, Vienna, Austria) with the EZR package (version 1.20; Saitama Medical Center, Jichi Medical University, Saitama, Japan). Values of $P < 0.05$ were considered significant.

Details on histological analysis and immunohistochemistry, immunofluorescence analysis, BrdU staining, RT-PCR analysis, and primary culture of intestinal fibroblasts are provided in Data S1.

### Results

**Activation status of ERK in normal intestines and intestinal polyps of Apc$^{Δ716}$ mice.** To study the roles of MEK/ERK signaling in intestinal polyp formation, we first determined the ERK activation status in normal intestines of WT C57BL/6 mice and intestinal adenomatous polyps in Apc$^{Δ716}$ mice. Western blot analysis with a p-ERK antibody showed ERK activation in the intestines of WT mice, with weaker activation in the colon compared with the small intestine (Fig. 1a). Immunohistochemical analysis showed marked ERK phosphorylation in intestinal epithelial cells of the crypts, which was stronger in the proximal parts of the small intestine, namely the duodenum and jejunum, as compared with the ileum and colon (Fig. 1b).

The ERK phosphorylation levels in intestinal stromal cells did not differ significantly in the proximal–distal axis of the intestines. One-week treatment of the WT mice with the MEK inhibitor trametinib greatly reduced the phosphorylation levels of ERK, but not those of JNK, p38 MAPK, STAT3, Akt, or S6, suggesting efficient and specific inhibition of the MEK/ERK pathway in the intestines by trametinib with minimal effects on the JNK, p38 MAPK, JAK–STAT, PI3K–Akt, or mTOR pathways (Fig. 1a–c). Western blot analysis using samples from 10-week old Apc$^{Δ716}$ mice showed similar levels of ERK phosphorylation between the normal intestinal mucosa and intestinal tumors (Fig. 1d). JNK signaling was activated in the tumors, whereas PI3K and p38 MAPK pathways were not, as we reported previously. Although ERK phosphorylation was observed in tumor epithelial cells near the luminal side, it was more prominent in tumor stromal cells (Fig. 1e). One-week treatment of Apc$^{Δ716}$ mice with trametinib significantly reduced the ERK phosphorylation level in the polyps (Fig. 1d, e). Trametinib treatment induced a mild increase in the p38 MAPK phosphorylation level in the polyps; the molecular mechanisms and biological significance underlying this observation are currently unknown.

To identify stromal cell types that showed MEK/ERK activation in the intestinal polyps of Apc$^{Δ716}$ mice, we next carried

![Fig. 2. Co-immunostaining analyses for p-ERK and markers of stromal cells. Immunofluorescence staining for p-ERK and vimentin (left panels, mesenchymal cell marker), CD34 (middle panels, vascular endothelial cell marker), and α-smooth muscle actin (α-SMA) (right panels, a myofibroblast marker) in small intestinal polyps of Apc$^{Δ716}$ mice using confocal laser scanning microscopy (×64). Nuclei are stained with DAPI (blue). Top panels show merged images with p-ERK in green, stromal cell markers in red, and DAPI in blue. Arrows indicate cells that are double-positive for p-ERK and stromal cell markers.](#)
out co-immunostaining with the p-ERK antibody and antibodies against markers of some stromal cell types, specifically vimentin (a mesenchymal cell marker), CD34 (a vascular endothelial cell marker), α-smooth muscle actin (α-SMA; a myofibroblast marker), β-tubulin III (a neuronal cell marker), lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1; a lymphatic endothelial cell marker), and CD45 (a leucocyte marker) (Figs 2,S1,S2). Although only a small fraction (<10%) of vimentin, CD34, or α-SMA positive cells showed co-staining for p-ERK in the normal intestinal mucosa (Fig. S1), a good percentage (>60%) of them showed co-staining in the polyps, especially in their luminal side (Figs 2,S1). The cells co-stained with both vimentin and p-ERK showed spindle-shaped morphology and their frequency was much higher than that of other specified stromal cells, which suggest that those cells are likely fibroblasts, although they are difficult to positively identify due to the lack of specific marker antibodies. We observed some stromal cells co-stained for p-ERK with β-tubulin III or LYVE-1. However, the frequency of β-tubulin III-positive or LYVE-1-positive cells was extremely low in the polyps (<10 per polyp section, as compared with >100 for vimentin-positive cells) and their significance is thus unknown (Figs S1,S2). Co-staining for p-ERK and CD45 was rarely observed in the polyps (Figs S1,S2). These results suggest that p-ERK-positive stromal cells in the ApcΔ716 polyps mainly include vascular endothelial cells, myofibroblasts, and probably fibroblasts.

**Trametinib treatment inhibits polyp formation in ApcΔ716 mice.**

To determine whether MEK/ERK signaling was involved in intestinal tumor formation, we then treated ApcΔ716 mice with trametinib for 8 weeks from 10 to 18 weeks of age (Fig. 3a). As shown in Figure 3(b,c), trametinib treatment significantly reduced the number of intestinal polyps in the large size class (Φ ≥ 1.5 mm). Polyps in trametinib-treated ApcΔ716 mice showed a collapsed morphology at the top (Fig. 3d), similarly to the polyps of ApcΔ716 mice in which COX-2 or mTORC1 was inhibited.(3,5,8)

To study the mechanism of inhibition of polyp formation by trametinib, we next evaluated cell proliferation rate by BrdU incorporation assay. Treatment with trametinib significantly reduced the BrdU labeling index of adenoma epithelial cells, whereas no change was observed in that of normal intestinal epithelial cells (Fig. 4a,b). Activation of ERK in tumor vascular endothelial cells (Fig. 2) suggests the possibility that MEK inhibition may affect tumor angiogenesis. We thus examined the effects of trametinib treatment on angiogenesis in ApcΔ716 intestinal polyps. Eight-week treatment significantly reduced the microvessel density in the polyps (Fig. 4c,d). These results suggest that trametinib treatment may suppress tumor formation in ApcΔ716 mice by inhibiting tumor cell proliferation and angiogenesis.

**Trametinib treatment reduces levels of COX-2 and CCL2 in ApcΔ716 polyps.** Stromal COX-2 has been implicated in intestinal polyp formation in ApcΔ716 mice through regulation of angiogenesis.(14) We next determined the effects of trametinib treatment on COX-2 expression levels. Western blot analysis showed that expression of COX-2, but not COX-1, was reduced in ApcΔ716 polyps as reported previously,(3) and that the COX-2 induction was suppressed dramatically by 1-week treatment with trametinib (Fig. 5a). Real-time RT-PCR analysis revealed that trametinib significantly decreased the COX-2 mRNA level in polyps (Fig. 5b). To address the possible roles
of the MEK/ERK signaling in COX-2 expression, we then examined localization of the COX-2 expressing cells and the cells positive for p-ERK staining in the polyps. As co-staining was not possible, we carried out immunohistochemical analysis using serial sections, which showed a similar staining pattern for p-ERK and COX-2 (Fig. 5c). Previous studies that showed the critical role of COX-2 in intestinal polyp formation in Apc<sup>D716</sup> mice identified stromal fibroblasts and vascular endothelial cells as the main cell types that expressed COX-2 in their tumors.<sup>(4)</sup> Because intestinal vascular endothelial cells were difficult to isolate for primary cultures, we examined the effects of trametinib treatment on COX-2 expression using primary culture of fibroblasts prepared from the intestines of ~8-week-old WT mice. Trametinib treatment strongly suppressed COX-2 induction by serum stimulation at both protein and mRNA levels (Fig. 5d,e), suggesting the requirement of MEK/ERK signaling for COX-2 expression in intestinal fibroblasts.

To gain insights into the possible mechanisms by which MEK/ERK signaling may contribute to tumor angiogenesis and tumor growth through COX-2 induction in intestinal fibroblasts, we carried out angiogenesis antibody array analysis using lysates of intestinal fibroblasts treated with rofecoxib or trametinib. As shown in Figure 5(f), treatment with rofecoxib or trametinib markedly reduced the level of CCL2 (MCP-1) as compared with vehicle control. Moreover, the mRNA level of Ccl2 was markedly elevated in the intestinal polyps of Apc<sup>D716</sup> mice, which was greatly reduced by trametinib treatment (Fig. 5g). Taken together, these results suggest the possibility that the MEK/ERK signaling positively regulates CCL2 expression through COX-2 induction in intestinal fibroblasts, which may help to promote angiogenesis and hence the growth of Apc<sup>D716</sup> polyps.

Discussion
In the present study, we have shown that the MEK inhibitor trametinib suppresses formation of intestinal adenomas in Apc<sup>D716</sup> mice, and propose that the antitumor effect of trametinib is at least in part mediated by its ability to suppress COX-2 induction in the tumor microenvironment. First, we have shown ERK activation not only in some tumor epithelial cells, but also in vascular endothelial cells, myofibroblasts, and vimentin-positive mesenchymal cells (likely fibroblasts) in the tumor stroma (Figs 1b,c,2,S1,S2). Second, trametinib treatment greatly reduced the COX-2 levels in Apc<sup>D716</sup> polyps <i>in vivo</i>, as well as in primary culture of fibroblasts prepared from mouse intestines <i>in vitro</i> (Fig. 5). Our previous studies on Apc<sup>D716</sup> mice clarified essential roles of COX-2 induction in intestinal tumor formation,<sup>(3,15,16)</sup> and identified fibroblasts and vascular endothelial cells as major producers of COX-2 in the intestinal polyps.<sup>(4)</sup> In this study, we have observed overlapped staining patterns for p-ERK-positive and COX-2-positive cells in serial sections of the luminal side of the polyps where most of the COX-2-expressing cells resided. Consistently, many vascular endothelial cells and fibroblasts were found in the luminal side of the polyps (Fig. S1).

Regarding the roles of ERK in intestinal tumor formation in Apc mutant mice, Lee <i>et al.</i><sup>(17)</sup> reported that intestinal microflora activated ERK in intestinal tumor epithelial cells of Apc<sup>Min</sup> mice through MyD88, a key signaling molecule

![Fig. 4. Trametinib attenuates adenoma cell proliferation and angiogenesis. (a) Micrographs of normal intestinal mucosa and adenoma epithelium labeled with BrdU in Apc<sup>D716</sup> mouse treated with vehicle (control) or trametinib for 8 weeks. Bar = 50 μm. (b) BrdU labeling indices of normal intestinal mucosa and adenoma epithelium in Apc<sup>D716</sup> mouse treated with vehicle (control) or trametinib. Data are average ± SD, and statistical significance was assessed by one-way ANOVA and Tukey's test. *P < 0.05. (c) Immunostaining of CD34 in a small intestinal polyp of vehicle-treated (left) and trametinib-treated (right) Apc<sup>D716</sup> mice. Bar = 50 μm. (d) Microvessel density in polyps of vehicle-treated (left) or trametinib-treated (right) Apc<sup>D716</sup> mice. Data are average ± SD, and statistical significance was assessed by Student's t-test. **P < 0.01.](image_url)
downstream of Toll-like receptors. They further showed that the activated ERK phosphorylated and thereby stabilized c-Myc protein in tumor epithelial cells, leading to promotion of the tumor growth.\(^{(17)}\) In the study they showed suppression of tumor formation in Apc\(^{Min}\) mice by treatment with the MEK1 inhibitor PD98059, consistent with our finding using trametinib. Although our present work suggests the roles of the MEK/ERK signaling in tumor microenvironment, in contrast to its direct role in tumor epithelial cells as suggested by Lee \textit{et al.}\(^{(17)}\), the two proposed mechanisms are not mutually exclusive and may co-operate in promoting tumor formation. The upstream signaling that activates ERK also remains to be clarified. Lee \textit{et al.}\(^{(17)}\) claimed the importance of intestinal bacteria in activating ERK through MyD88 in intestinal epithelial cells. However, experiments using germ-free mice or antibiotic treatment showed mixed results as to the roles of intestinal microflora in tumor formation in Apc mutant mice.\(^{(18–22)}\) with some reports suggesting their limited roles in tumor formation.

Angiogenesis antibody array analysis identified CCL2 (also known as MCP-1) as a candidate molecule regulated by both MEK/ERK signaling and COX-2 in intestinal fibroblasts (Fig. 5f). CCL2 is a pro-inflammatory chemokine that has been implicated in monocyte recruitment, angiogenesis, and development of tumor microenvironment.\(^{(23)}\) Previous studies by others showed that Ccl2 was highly expressed in the polyps of Apc\(^{Min}\) mice,\(^{(24)}\) and that Ccl2 deficiency significantly reduced the number of intestinal polyps, especially that of large polyps, in Apc\(^{Min}\) mice.\(^{(25)}\) We confirmed elevated Ccl2 mRNA levels in the polyps of Apc\(^{D716}\) mice, which were markedly reduced by trametinib treatment (Fig. 5g), suggesting the possibility that the MEK/ERK signaling activation in stromal fibroblasts may promote tumor angiogenesis in part by positively regulating COX-2 and CCL2. We also observed co-staining of p-ERK and vascular endothelial cells in intestinal tumors (Fig. 2). MEK/ERK signaling has been implicated in angiogenesis;\(^{(26)}\) for instance, the MEK1 inhibitor selumetinib was shown to suppress the growth of non-small cell lung cancer
stromal fibroblasts. The antiangiogenic activity of trametinib could thus be combination of direct effects on endothelial cells and indirect effects mediated by reduced expression of COX-2 in fibroblasts.

The mechanisms by which MEK/ERK signaling positively regulates COX-2 in stromal fibroblasts and vascular endothelial cells of Apc\(^{−/−}\) polyps are currently unknown. Expression of COX-2 is regulated at the transcriptional and post-transcriptional levels, and ERK has been implicated in both levels of control in fibroblasts. The molecular mechanism of the MEK/ERK activation in the tumor stroma are also not clear. Further studies will elucidate the molecular mechanisms underlying COX-2 induction by MEK/ERK signaling in tumor stromal fibroblasts. 

Trametinib has been approved for treating metastatic melanoma patients with BRAFV600E/K mutations. Studies on metastatic melanoma patients with BRAFV600E/K mutations showed that combination treatment with the BRAF inhibitor dabrafenib and trametinib had better efficacy than single treatments showed that combination treatment with the BRAF inhibitor trametinib when combined with BRAF inhibitors thus may, in part, be through its effect on reducing COX-2 levels. Clinical trials are underway for treatment of colorectal cancers using multiple kinase inhibitors including trametinib and dabrafenib. ClinicalTrials.gov identifiers NCT01750918 and NCT01902171, in which the ability of trametinib to reduce COX-2 levels may also be beneficial.

In conclusion, we have shown that MEK/ERK signaling is activated in some tumor epithelial cells and stromal cells in the intestinal polyps of Apc\(^{−/−}\) mice, and that the MEK inhibitor trametinib potently suppresses tumor formation, accompanied by reduced COX-2 induction in the tumor stroma. These results also suggest that trametinib, a drug that is already approved for treating a subset of melanoma patients, may be useful for prevention of colon polyp formation in familial adenomatous polyposis patients with Apc mutations.

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Disclosure Statement

The authors have no conflict of interest.
Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Materials and methods.

Fig. S1. Co-immunostaining analyses for identification of stromal cells that show ERK activation.

Fig. S2. Co-immunostaining analyses for p-ERK and selected stromal cell markers.