TITLE:
Antitumor activity of the MEK inhibitor trametinib on intestinal polyp formation in Apc[Δ716] mice involves stromal COX-2

AUTHOR(S):
Fujishita, Teruaki; Kajino-Sakamoto, Rie; Kojima, Yasushi; Taketo, Makoto Mark; Aoki, Masahiro

CITATION:
Fujishita, Teruaki ...[et al]. Antitumor activity of the MEK inhibitor trametinib on intestinal polyp formation in Apc[Δ716] mice involves stromal COX-2. Cancer Science 2015, 106(6): 692-699

ISSUE DATE:
2015-06

URL:
http://hdl.handle.net/2433/215164

RIGHT:
© 2015 The Authors. Cancer Science published by Wiley Publishing Asia Pty Ltd on behalf of Japanese Cancer Association. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
Antitumor activity of the MEK inhibitor trametinib on intestinal polypl formation in Apc<sup>Δ716</sup> mice involves stromal COX-2

Teruki Fujishita, Rie Kajino-Sakamoto, Yasushi Kojima, Makoto Mark Taketo and Masahiro Aoki

Key words
Adenomatous polyposis coli, chemokine CCL2, colorectal neoplasms, cyclooxygenase-2, extracellular signal-regulated MAP kinases

Correspondence
Masahiro Aoki, Division of Molecular Pathology, Aichi Cancer Center Research Institute, Nagoya; Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Funding Information
Japan Society for the Promotion of Science (Kakenhi) (24790382, 26290045); Yasuda Medical Foundation; Suzuken Memorial Foundation; Takeda Science Foundation.

Received January 2, 2015; Revised March 23, 2015; Accepted April 3, 2015

Cancer Sci 106 (2015) 692-699
doi: 10.1111/cas.12670

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. 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. 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. We subsequently found that JNK activation in adenoma epithelial cells of Apc mutant mice was responsible for mTORC1 activation and hence tumor formation. Extracellular signal-regulated kinase is a 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<sup>Δ716</sup> 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<sup>Δ716</sup> 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<sup>Δ716</sup> 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 polytumours, in intestinal fibroblasts in vitro. Consistently, trametinib treatment reduced the Ccl2 mRNA level in Apc<sup>Δ716</sup> tumors in vivo. These results suggest that MEK/ERK signaling plays key roles in intestinal adenoma formation in Apc<sup>Δ716</sup> mice, at least in part, through COX-2 induction in tumor stromal cells.

Materials and Methods

Animals and drug treatments. The construction of Apc<sup>Δ716</sup> mice was described previously. Male and female Apc<sup>Δ716</sup> 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.

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 β-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,
then cultured in DMEM with 10% FBS in the presence of trametinib (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\(^{D716}\) 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\(^{D716}\) 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\(^{D716}\) 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\(^{D716}\) 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\(^{D716}\) mice, we next carried out a series of experiments to determine the roles of MEK/ERK signaling in the development of intestinal polyps. Immunofluorescence staining for p-ERK and markers of stromal cells was carried out to identify the cell types that were activated by MEK/ERK signaling. Co-immunostaining analyses for p-ERK and markers of stromal cells (Fig. 2) showed that trametinib suppressed intestinal polyps.

![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\(^{D716}\) 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.](https://repository.kulib.kyoto-u.ac.jp)
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 suggests 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 Apc<sup>D716</sup> polyps mainly include vascular endothelial cells, myofibroblasts, and probably fibroblasts.

**Trametinib treatment inhibits polyp formation in Apc<sup>D716</sup> mice.**

To determine whether MEK/ERK signaling was involved in intestinal tumor formation, we then treated Apc<sup>D716</sup> 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 Apc<sup>D716</sup> mice showed a collapsed morphology at the top (Fig. 3d), similarly to the polyps of Apc<sup>D716</sup> mice in which COX-2 or mTORC1 was inhibited.

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 Apc<sup>D716</sup> 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 Apc<sup>D716</sup> mice by inhibiting tumor cell proliferation and angiogenesis.

**Trametinib treatment reduces levels of COX-2 and CCL2 in Apc<sup>D716</sup> polyps.**

Stromal COX-2 has been implicated in intestinal polyp formation in Apc<sup>D716</sup> mice through regulation of angiogenesis. 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 induced in Apc<sup>D716</sup> polyps as reported previously, 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. S3). Previous studies that showed the critical role of COX-2 in intestinal polyp formation in Apc\(^{\Delta716}\) mice identified stromal fibroblasts and vascular endothelial cells as the main cell types that expressed COX-2 in their tumors.\(^{(4)}\) 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 in 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\(^{\Delta716}\) 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\(^{\Delta716}\) polyps.

Discussion

In the present study, we have shown that the MEK inhibitor trametinib suppresses formation of intestinal adenomas in Apc\(^{\Delta716}\) 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\(^{\Delta716}\) polyps in vivo, as well as in primary culture of fibroblasts prepared from mouse intestines in vitro (Fig. 5). Our previous studies on Apc\(^{\Delta716}\) mice clarified essential roles of COX-2 induction in intestinal tumor formation\(^{(3,15,16)}\) and identified fibroblasts and vascular endothelial cells as major producers of COX-2 in the intestinal polyps.\(^{(4)}\) 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 et al.\(^{(17)}\) reported that intestinal microflora activated ERK in intestinal tumor epithelial cells of Apc\(^{Min}\) mice through MyD88, a key signaling molecule.
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 \(\text{Apc}^{\text{Min}}\) 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 \(\text{et al.}\),\(^{(17)}\) 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 \(\text{et al.}\)\(^{(17)}\) 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 \(\text{Apc}^{\text{Min}}\) 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 \(\text{Apc}^{\text{Min}}\) mice,\(^{(24)}\) and that Ccl2 deficiency significantly reduced the number of intestinal polyps, especially that of large polyps, in \(\text{Apc}^{\text{Min}}\) mice.\(^{(25)}\) We confirmed elevated Ccl2 mRNA levels in the polyps of \(\text{Apc}^{\text{D716}}\) 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...
xenografts, accompanied by decreased microvessel density. 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 Apc716+/− 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.(19,10) 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 dabrafenib treatment, and also induced less skin toxicity, with decreased numbers of patients developing hyperkeratosis or skin papillomas.(33) A recent report showed that COX-2 inhibition decreases microvessel density.(27) A recent report showed that COX-2 inhibition can prevent the appearance of keratoacanthomas and cutaneous squamous cell carcinomas after treatment with BRAF inhibitors in a mouse model.(34) The beneficial effects of 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 Apc716+/− 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.

Acknowledgments
This work was supported by the Japan Society for the Promotion of Science (Kakenhi grant no. 24790382 to T.F. and grant no. 26290045 to M.A.). This research was also supported by The Yasuda Medical Foundation, Suzuken Memorial Foundation (to T.F.), and Takeda Science Foundation (to M.A.). The authors thank R. Mitsuya, Y. Fuma, and Y. Goto for their technical assistance.

Disclosure Statement
The authors have no conflict of interest.

References
1 Aoki M, Taketo MM. Use of genetically engineered mouse models in identification and validation of therapeutic targets for colon cancer. In: Goss KH, Kahn M, eds. Targeting the Wnt Pathway in Cancer. New York: Springer; 2011: 143–63.
2 Oshima M, Oshima H, Kitagawa K et al. Loss of ApC heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated ApC gene. Proc Natl Acad Sci U S A 1995; 92: 4482–6.
3 Oshima M, Dinchuk JE, Kargman SL et al. Suppression of intestinal polypogenesis in ApC716 mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996; 87: 803–9.
4 Sonoshita M, Takaku K, Oshima M, Sugihara K, Taketo MM. Cyclooxygenase-2 expression in fibroblasts and endothelial cells of intestinal polyps. Cancer Res 2001; 61: 6848–9.
5 Fujisita T, Aoki K, Lane HA, Aoki M, Taketo MM. Inhibition of the mTORC1 pathway suppresses intestinal polyp formation and reduces mortality in ApC716−/− mice. Proc Natl Acad Sci U S A 2008; 105: 13544–50.
6 Koehl GE, Spitzner M, Ousingsawat J, Schreiber R, Geissler EK, Kunzelmann E. Tapamycin inhibits oncogenic intestinal ion channels and neoplasia in Apc min/+ mice. Oncogene 2010; 29: 1553–60.
7 Metcalfe C, Ibrahim AE, Graeb M et al. Dvl2 promotes intestinal length and neoplasia in the Apc+/− mouse model for colorectal cancer. Cancer Res 2010; 70: 6629–38.
8 Fujisita T, Aoki M, Taketo MM. JNK signaling promotes intestinal tumoriogenesis through activation of mTORC1 complex 1 in Apc716+/− mice. Gastroenterology 2011; 140: 1556–65.
9 Akinleye A, Furqan M, Mukhi N, Ravella P, Liu D. MEK and the inhibitors: Tpl2 ablation promotes intestinal tumorigenesis in Apc min/− mice. Proc Natl Acad Sci U S A 2012; 109: E1082–91.
10 Borsig L, Wolf MJ, Roblek M, Heikenwalder M. Inflammatory chemokines and microbial products drive IL-17-mediated tumour growth. Nature 2012; 491: 254–8.
11 Serebrennikova OB, Tsatsanis C, Mao C et al. Tpl2 ablation promotes intestinal tumoural proliferation and tumorigenesis in Apc min/− mice by inhibiting IL-10 secretion and regulatory T-cell generation. Proc Natl Acad Sci U S A 2012; 109: E1082–91.
12 McCullenn JL, Davis JM, Steiner JL et al. Intestinal inflammatory cytokine response in relation to tumorigenesis in the ApC716 mouse. Cytokeine 2012; 47: 113–9.
13 McCullenn JL, Davis JM, Steiner JL et al. Linking tumor-associated macrophages, inflammation, and intestinal tumorigenosis: role of MCP-1. Am J Physiol Gastrointest Liver Physiol 2012; 303: G1087–95.
14 D’Angelo G, Strumian I, Martial J, Weiner RJ. Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin. Proc Natl Acad Sci U S A 1995; 92: 6374–8.
15 Takahashi O, Komaki R, Smith PD et al. Combined MEK and VEGFR inhibition in orthotopic human lung cancer models results in enhanced inhibition of tumor growth, angiogenesis, and metastasis. Clin Cancer Res 2012: 18: 1641–54.
16 Wang D, DuBois RN. The role of COX-2 in intestinal inflammation and colorectal cancer. Oncogene 2010; 29: 781–8.
29 Xie W, Herschman H. Transcriptional regulation of prostaglandin synthase 2 gene expression by platelet-derived growth factor and serum. J Biol Chem 1996; 271: 31742–8.

30 Sheng H, Williams S, Shao J, Liang P, DuBois RN, Beauchamp RD. Induction of cyclooxygenase-2 by activated Ha-ras oncogene in Rat-1 fibroblasts and the role of mitogen-activated protein kinase pathway. J Biol Chem 1998; 273: 22120–7.

31 Frungieri MB, Albrecht M, Raemsch R, Mayerhofer A. The action of the mast cell product tryptase on cyclooxygenase-2 (COX2) and subsequent fibroblast proliferation involves activation of the extracellular signal-regulated kinase isoforms 1 and 2 (erk1/2). Cell Signal 2005; 17: 525–33.

32 Flaherty KR, Robert C, Hersey P et al. Improved survival with MEK inhibition in BRAF-mutated melanoma. N Engl J Med 2012; 367: 107–14.

33 Holderfield M, Nagel TE, Stuart DD. Mechanism and consequence of RAF kinase activation by small-molecule inhibitors. Br J Cancer 2014; 111: 640–5.

34 Escuin-Ordinas H, Atefi M, Fu Y et al. COX-2 inhibition prevents appearance of cutaneous squamous cell carcinomas accelerated by BRAF inhibitors. Mol Oncol 2014; 8: 250–60.

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.