4. Discussion

MAPO1 was identified as one of the protein elements functioning at a certain step following the induction of apoptosis [16]. In *Mapo1*-defective cells, mitochondrial membrane depolarization and caspase-3 activation were not observed even after exposure to MNU, although the cells retain the ability for mismatch repair protein-dependent DNA damage detection and signaling. Subsequent studies have revealed that MAPO1 is identical to FNIP2 and FNIPL, reported by Hasumi et al. [23] and Takagi et al. [24], respectively. This protein is bound to folliculin, encoded by the *FLCN* tumor suppressor gene, and AMP-activated protein kinase (AMPK). To analyze the possible roles of folliculin and AMPK in the induction of apoptosis, we introduced siRNAs specific for the *Flcn* or *Ampkα* gene and then treated the cells with MNU. The flow cytometric analyses performed to measure the sub-G_1 population of cells revealed that folliculin and AMPK, as well as MAPO1, were involved in MNU-induced apoptosis. Taken together, these data suggest that MAPO1 forms a protein complex(es) with folliculin and AMPK, and plays a role in a signal transduction pathway of apoptosis.

It is known that AMPK is one of the signaling kinases that negatively regulates cell growth and proliferation and is phosphorylated itself under conditions of energetic stress [26–29]. Several recent papers have observed the pro-apoptotic potential of activated AMPK [30–33]. In this report, we found a gradual increase in the levels of AMPK phosphorylation in *Mapo1*-proficient cells after MNU treatment, implying a possible involvement of the activation of AMPK in the MNU-induced apoptosis pathway. In *Mapo1*-deficient cells, AMPK activation in this manner was hardly detectable, even after the treatment with MNU. Furthermore, the treatment of cells with AICAR, a specific activator of AMPK, resulted in AMPKα phosphorylation and mitochondrial membrane depolarization in a *Mapo1*-dependent manner. These findings extended onto the case of *Flcn*-knockdown cells. Taken together, it is likely that MAPO1 and FLCN positively regulate the activation of AMPK through their mutual interaction in the apoptotic signaling pathway, triggered by an alkylating agent. MAPO1 and FLCN proteins have been reported to undergo some modifications in cells [17,24]. The treatment with an alkylating agent might affect the modified states of these proteins, and might cause the activation of the protein complex, thus leading to AMPK activation. Another folliculin-interacting protein, FNIP1, which is homologous to MAPO1, is also capable of binding to AMPK [17]. The activation of AMPK might therefore be regulated in more complex ways under the balance of MAPO1 and FNIP1 activities.

Another important problem which remains to be solved is how the AMPK–MAPO1–FLCN complex is activated by the signal delivered from the mismatch repair protein complex, which itself is activated through the interaction with DNA carrying base mismatches. The signal may be delivered by direct physical contact between the two complexes or through the involvement of other protein factors. The protein linking analyses, aided by mass spectrometry, have been performed, but no evidence to show the physical association of the two complexes was obtained (unpublished results). It seems likely, therefore, that some other protein factor(s) might be involved in the signal transduction process. To identify such factors, it would be relevant to extend this approach using retrovirus-mediated gene-trap mutagenesis studies.

Germline mutations in the *FLCN* gene have been identified in patients with Birt-Hogg-Dubé (BHD) syndrome, which is an autosomal dominant disorder characterized by hamartomas of skin follicles, spontaneous pneumothorax, and renal tumors [20–22]. Furthermore, *BHD* heterozygous knockout mice were revealed to develop kidney cysts and tumors as they aged, while *BHD* homozygous null mice displayed early embryonic lethality [34,35]. The recent findings, including this report, strongly suggest that folliculin has physical and/or functional interactions with the AMPK-mTOR signaling pathway [17,34,36]. Mutations in several other tumor suppressor genes, such as *LKB1*, *TSC1* and *TSC2* [29,37], have also been shown to lead to dysregulation of AMPK-mTOR signaling and to the development of other hamartomatous syndromes. Our present findings that folliculin is involved in the induction of apoptosis might shed some light on the physiological roles of *BHD/FLCN* and other related tumor suppressor genes. We are currently establishing *Mapo1* knockout mice to analyze the possible roles of the gene in the suppression of tumor predisposition resulting from environmental stresses.

Conflict of interest statement

The authors declare that there are no conflicts of interests.

Acknowledgments

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References

[1] D.T. Beranek, Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents, Mutat. Res. 231 (1990) 11–30.
[2] C. Coulondre, J.H. Miller, Genetic studies of the lac repressor. IV. Mutagenic specificity in the lacI gene of *Escherichia coli*, J. Mol. Biol. 117 (1977) 577–606.
[3] T. Ito, T. Nakamura, H. Maki, M. Sekiguchi, Roles of transcription and repair in alkylation mutagenesis, Mutat. Res. 314 (1994) 273–285.
[4] B. Demple, A. Jacobsson, M. Olsson, P. Robins, T. Lindahl, Repair of alkylated DNA in *Escherichia coli*. Physical properties of O6-methylguanine–DNA methyltransferase, J. Biol. Chem. 257 (1982) 13776–13780.
[5] H. Kawate, K. Ihara, K. Kohda, K. Sakumi, M. Sekiguchi, Mouse methyltransferase for repair of O6-methylguanine and O4-methylthymine in DNA, Carcinogenesis 16 (1995) 1595–1602.
[6] P. Branch, G. Aquilina, M. Bignami, P. Karran, Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage, Nature 362 (1993) 652–654.
[7] M. Hidaka, Y. Takagi, T.Y. Takano, M. Sekiguchi, PCNA-MutSalpha-mediated binding of MutLalpha to replicative DNA with mismatched bases to induce apoptosis in human cells, Nucleic Acids Res. 33 (2005) 5703–5712.
[8] A. Kat, W.G. Thilly, W.H. Fang, M.J. Longley, G.M. Li, P. Modrich, An alkylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 6424–6428.
[9] B.J. Glassner, G. Weeda, J.M. Allan, J.L. Broekhof, N.H. Carls, I. Donker, B.P. Engelward, R.J. Hampson, R. Hersmus, M.J. Hickman, R.B. Roth, H.B. Warren, M.M. Wu, J.H. Hoeijmakers, L.D. Samson, DNA repair methyltransferase (Mgmt) knockout mice are sensitive to the lethal effects of chemotherapeutic alkylating agents, Mutagenesis 14 (1999) 339–347.
[10] K. Sakumi, A. Shiraishi, S. Shimizu, T. Tsuzuki, T. Ishikawa, M. Sekiguchi, Methylnitrosourea-induced tumorigenesis in MGMT gene knockout mice, Cancer Res. 57 (1997) 2415–2418.
[11] A. Shiraishi, K. Sakumi, M. Sekiguchi, Increased susceptibility to chemotherapeutic alkylating agents of mice deficient in DNA repair methyltransferase, Carcinogenesis 21 (2000) 1879–1883.
[12] T. Tsuzuki, K. Sakumi, A. Shiraishi, H. Kawate, H. Igarashi, T. Iwakuma, Y. Tominaga, S. Zhang, S. Shimizu, T. Ishikawa, et al., Targeted disruption of the DNA repair methyltransferase gene renders mice hypersensitive to alkylating agent, Carcinogenesis 17 (1996) 1215–1220.
[13] H. Kawate, K. Sakumi, T. Tsuzuki, Y. Nakatsuru, T. Ishikawa, S. Takahashi, H. Takano, T. Noda, M. Sekiguchi, Separation of killing and tumorigenic effects of an alkylating agent in mice defective in two of the DNA repair genes, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 5116–5120.
[14] Y. Takagi, M. Takahashi, M. Sanada, R. Ito, M. Yamaizumi, M. Sekiguchi, Roles of MGMT and MLH1 proteins in alkylation-induced apoptosis and mutagenesis, DNA Repair (Amst.) 2 (2003) 1135–1146.
[15] K. Ochs, B. Kaina, Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/Caspase-8 independent, Cancer Res. 60 (2000) 5815–5824.
[16] K. Komori, Y. Takagi, M. Sanada, T.H. Lim, Y. Nakatsu, T. Tsuzuki, M. Sekiguchi, M. Hidaka, A novel protein, MAPO1, that functions in apoptosis triggered by O6-methylguanine mispair in DNA, Oncogene 28 (2009) 1142–1150.
[17] M. Baba, S.B. Hong, N. Sharma, M.B. Warren, M.L. Nickerson, A. Iwamatsu, D. Esposito, W.K. Gillette, R.F. Hopkins 3rd, J.L. Hartley, M. Furihata, S. Oishi, W. Zhen, T.R. Burke, W.M. Linehan Jr., L.S. Schmidt, B. Zbar, Folliculin encoded

by the BHD gene interacts with a binding protein, FNIP1, and AMPK and is involved in AMPK and mTOR signaling, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 15552–15557.
[18] M.L. Nickerson, M.B. Warren, J.R. Toro, V. Matrosova, G. Glenn, M.L. Turner, P. Duray, M. Merino, P. Choyke, C.P. Pavlovich, N. Sharma, M. Walther, D. Munroe, R. Hill, E. Maher, C. Greenberg, M.I. Lerman, W.M. Linehan, B. Zbar, L.S. Schmidt, Mutations in a novel gene lead to kidney tumors, lung wall defects, and benign tumors of the hair follicle in patients with the Birt-Hogg-Dube syndrome, Cancer Cell 2 (2002) 157–164.
[19] C.D. Vocke, Y. Yang, C.P. Pavlovich, L.S. Schmidt, M.L. Nickerson, C.A. Torres-Cabala, M.J. Merino, M.M. Walther, B. Zbar, W.M. Linehan, High frequency of somatic frameshift BHD gene mutations in Birt-Hogg-Dube-associated renal tumors, J. Natl. Cancer Inst. 97 (2005) 931–935.
[20] A.R. Birt, G.R. Hogg, W.J. Dube, Hereditary multiple fibrofolliculomas with trichodiscomas and acrochordons, Arch. Dermatol. 113 (1977) 1674–1677.
[21] J.R. Toro, G. Glenn, P. Duray, T. Darling, G. Weirich, B. Zbar, M. Linehan, M.L. Turner, Birt-Hogg-Dube syndrome: a novel marker of kidney neoplasia, Arch. Dermatol. 135 (1999) 1195–1202.
[22] B. Zbar, W.G. Alvord, G. Glenn, M. Turner, C.P. Pavlovich, L. Schmidt, M. Walther, P. Choyke, G. Weirich, S.M. Hewitt, P. Duray, F. Gabril, C. Greenberg, M.J. Merino, J. Toro, W.M. Linehan, Risk of renal and colonic neoplasms and spontaneous pneumothorax in the Birt-Hogg-Dube syndrome, Cancer Epidemiol. Biomarkers Prev. 11 (2002) 393–400.
[23] H. Hasumi, M. Baba, S.B. Hong, Y. Hasumi, Y. Huang, M. Yao, V.A. Valera, W.M. Linehan, L.S. Schmidt, Identification and characterization of a novel folliculin-interacting protein FNIP2, Gene 415 (2008) 60–67.
[24] Y. Takagi, T. Kobayashi, M. Shiono, L. Wang, X. Piao, G. Sun, D. Zhang, M. Abe, Y. Hagiwara, K. Takahashi, O. Hino, Interaction of folliculin (Birt-Hogg-Dube gene product) with a novel Fnip1-like (FnipL/Fnip2) protein, Oncogene 27 (2008) 5339–5347.
[25] D. Carling, The AMP-activated protein kinase cascade – a unifying system for energy control, Trends Biochem. Sci. 29 (2004) 18–24.
[26] D.G. Hardie, The AMP-activated protein kinase pathway – new players upstream and downstream, J. Cell Sci. 117 (2004) 5479–5487.
[27] S.A. Hawley, M. Davison, A. Woods, S.P. Davies, R.K. Beri, D. Carling, D.G. Hardie, Characterization of the AMP-activated protein kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase, J. Biol. Chem. 271 (1996) 27879–27887.
[28] J.M. Lizcano, O. Goransson, R. Toth, M. Deak, N.A. Morrice, J. Boudeau, S.A. Hawley, L. Udd, T.P. Makela, D.G. Hardie, D.R. Alessi, LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1, EMBO J. 23 (2004) 833–843.
[29] R.J. Shaw, M. Kosmatka, N. Bardeesy, R.L. Hurley, L.A. Witters, R.A. DePinho, L.C. Cantley, The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 3329–3335.
[30] C. Cao, S. Lu, R. Kivlin, B. Wallin, E. Card, A. Bagdasarian, T. Tamakloe, W.M. Chu, K.L. Guan, Y. Wan, AMP-activated protein kinase contributes to UV- and H2O2-induced apoptosis in human skin keratinocytes, J. Biol. Chem. 283 (2008) 28897–28908.
[31] R.G. Jones, D.R. Plas, S. Kubek, M. Buzzai, J. Mu, Y. Xu, M.J. Birnbaum, C.B. Thompson, AMP-activated protein kinase induces a p53-dependent metabolic checkpoint, Mol. Cell 18 (2005) 283–293.
[32] R. Okoshi, T. Ozaki, H. Yamamoto, K. Ando, N. Koida, S. Ono, T. Koda, T. Kamijo, A. Nakagawara, H. Kizaki, Activation of AMP-activated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress, J. Biol. Chem. 283 (2008) 3979–3987.
[33] W.B. Zhang, Z. Wang, F. Shu, Y.H. Jin, H.Y. Liu, Q.J. Wang, Y. Yang, Activation of AMP-activated protein kinase by temozolomide contributes to apoptosis in glioblastoma cells via p53 activation and mTORC1 inhibition, J. Biol. Chem. 285 (2010) 40461–40471.
[34] T.R. Hartman, E. Nicolas, A. Klein-Szanto, T. Al-Saleem, T.P. Cash, M.C. Simon, E.P. Henske, The role of the Birt-Hogg-Dube protein in mTOR activation and renal tumorigenesis, Oncogene 28 (2009) 1594–1604.
[35] Y. Hasumi, M. Baba, R. Ajima, H. Hasumi, V.A. Valera, M.E. Klein, D.C. Haines, M.J. Merino, S.B. Hong, T.P. Yamaguchi, L.S. Schmidt, W.M. Linehan, Homozygous loss of BHD causes early embryonic lethality and kidney tumor development with activation of mTORC1 and mTORC2, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 18722–18727.
[36] X. Piao, T. Kobayashi, L. Wang, M. Shiono, Y. Takagi, G. Sun, M. Abe, Y. Hagiwara, D. Zhang, K. Okimoto, M. Kouchi, I. Matsumoto, O. Hino, Regulation of folliculin (the BHD gene product) phosphorylation by Tsc2-mTOR pathway, Biochem. Biophys. Res. Commun. 389 (2009) 16–21.
[37] K. Inoki, M.N. Corradetti, K.L. Guan, Dysregulation of the TSC-mTOR pathway in human disease, Nat. Genet. 37 (2005) 19–24.

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REVIEW

The role of PGE_2-associated inflammatory responses in gastric cancer development

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Abstract Accumulating evidence indicates that inflammation plays a critical role in cancer development. Cyclooxygenase-2 (COX-2) is a rate-limiting enzyme for prostanoid biosynthesis, including prostaglandin E_2 (PGE_2), and plays a key role in both inflammation and cancer. It has been demonstrated that inhibition of COX-2 and PGE_2 receptor signaling results in the suppression of tumor development in a variety of animal models. However, the molecular mechanisms underlying COX-2/PGE_2-associated inflammation in carcinogenesis have not yet been fully elucidated. In order to study the role of PGE_2-associated inflammatory responses in tumorigenesis, it is important to use in vivo mouse models that recapitulate human cancer development from molecular mechanisms with construction of tumor microenvironment. We have developed a gastritis model (*K19-C2mE* mice) in which an inflammatory microenvironment is constructed in the stomach via induction of the COX-2/PGE_2 pathway. We also developed a gastric cancer mouse model (*Gan* mice) in which the mice develop inflammation-associated gastric tumors via activation of both the COX-2/PGE_2 pathway and Wnt signaling. Expression analyses using these in vivo models have revealed novel mechanisms of the inflammatory responses underlying gastric cancer development. PGE_2-associated inflammatory responses activate epidermal growth factor receptor (EGFR) signaling through the induction of EGFR ligands and ADAMs that release EGFR ligands from the cell membrane. In *Gan* mice, a combination treatment with EGFR and COX-2 inhibitors significantly suppresses gastric tumorigenesis. Moreover, PGE_2-associated inflammation downregulates tumor suppressor microRNA, miR-7, in gastric cancer cells, which suppresses epithelial differentiation. These results indicate that PGE_2-associated inflammatory responses promote in vivo gastric tumorigenesis via several different molecular mechanisms.

Keywords Gastric cancer · Inflammation · COX-2 · PGE_2 · EGFR · MicroRNA

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Introduction

It has been shown that approximately 15–20 % of malignant cancers are associated with chronic infection [1, 2]. For example, infection with *Helicobacter pylori*, hepatitis C virus, and human papilloma virus is closely associated with the development of gastric cancer, hepatocellular carcinoma, and cervical cancer, respectively. On the other hand, it has also been reported that tobacco smoke and obesity contribute to tumor development through induction of inflammatory responses in the lungs and liver, respectively [3, 4]. These results, together with those of recent genetic studies, indicate that inflammatory responses play important roles in cancer development regardless of whether they are caused by infectious or noninfectious stimuli [5–7]. Among the various inflammatory networks involved in tumor microenvironment, the cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2) pathway was first identified as a key player in tumorigenesis [8, 9]. COX-2 is an inducible rate-limiting enzyme for prostaglandin biosynthesis and has been shown to play an essential role in inflammatory responses. PGE_2 is a downstream product of COX-2, and the level of PGE_2 increases significantly in tumor tissues, suggesting that PGE_2 plays a role in

tumorigenesis. Although almost 20 years have passed since the discovery of the role of COX-2 in cancer development, the mechanisms underlying the role of the COX-2/PGE_2 pathway in tumorigenesis have not yet been fully elucidated. It was recently shown that COX-2 expression and PGE_2 production are induced in mesenchymal stem cells infiltrated in cancer tissues, which leads to the expression of inflammatory cytokines and the induction of epithelial mesenchymal transition through the construction of niches for tumor-initiating cells [10]. However, it remains unclear how the COX-2/PGE_2 pathway promotes in vivo tumorigenesis. To understand the role of PGE_2-induced inflammatory responses in cancer development, it is important to construct animal models in which the animals develop cancer caused by oncogenic activation in epithelial cells together with PGE_2-associated host responses like human cancers. We developed a gastric cancer mouse model (*Gan* mice for *ga*stric *n*eoplasia mice) in which activation of both Wnt signaling and the COX-2/PGE_2 pathway in the stomach is achieved by the transgenic expression of *Wnt1*, *Ptgs2*, and *Ptges*, which encode Wnt1, COX-2, and mPGES-1, respectively [11, 12]. By conducting expression analyses of mRNA and microRNA (miRNA) using murine gastritis tissues and *Gan* mouse gastric tumors, we identified novel molecular pathways that are activated or suppressed in PGE_2-associated inflammatory microenvironment and thereby contribute to gastric cancer development. In the first part of this article, we review the role of the COX-2/PGE_2 pathway in gastrointestinal cancer development and the construction of the *Gan* mouse model. Then, we discuss the novel mechanisms underlying inflammation in gastric tumorigenesis, which we found using the *Gan* mouse model system.

The COX-2/PGE_2 pathway in gastrointestinal tumor development

Approximately 20 years ago, epidemiological studies reported that the regular use of non-steroidal anti-inflammatory drugs (NSAIDs) lowers the mortality rate of patients with gastrointestinal cancer [13, 14]. The target molecules of NSAIDs are COX-1 and COX-2, which are rate-limiting enzymes for prostaglandin biosynthesis. COX-1 is constitutively expressed and plays a physiological house-keeping role, while COX-2 expression is induced in both inflammation and cancer. It has also been reported that treatment of patients with familial adenomatous polyposis (FAP) using NSAIDs results in significant decreases in the number and size of colon polyps [15]. These results strongly suggested that COX-2 plays an important role in intestinal tumorigenesis. Moreover, a large number of animal experiments have confirmed that NSAID treatment suppresses chemical carcinogen-induced colon tumor development, indicating that induction of the COX pathway is required for tumor development [16]. Mouse genetic studies clearly demonstrate the role of COX-2 in intestinal tumorigenesis. Disruption of the *Ptgs2* gene in $Apc^{\Delta 716}$ and Apc^{Min} mice, mouse models for FAP, significantly suppresses the development of intestinal polyposis, thus indicating an essential role for COX-2 in tumor development [17, 18]. Interestingly, disruption of *Ptgs1* (COX-1 gene) also suppresses intestinal polyposis in Apc^{Min} mice. It is therefore possible that the COX-1-derived basal level of PGE_2 is also involved in tumor formation before COX-2 expression is induced [16] (Fig. 1).

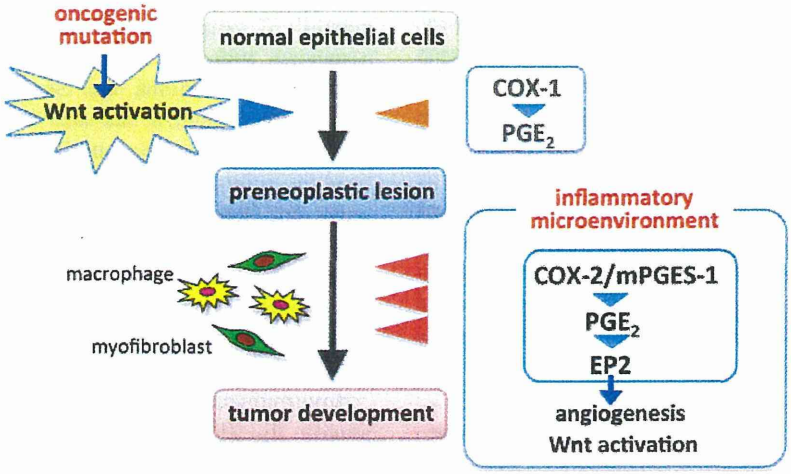

Fig. 1 Schematic diagram showing the role of PGE_2 in tumorigenesis. Wnt signaling activation triggers initiation of tumorigenesis. Expression of COX-2 and mPGES-1 is induced in microadenomas, which results in an increase of PGE_2 level. Activated COX-2/PGE_2 pathway contributes to tumor development through a variety of mechanisms, including induction of angiogenesis and Wnt activation, or construction of inflammatory microenvironment as indicated in Figs. 4 and 5. COX-1-derived PGE_2 may play a role in tumorigenesis at the early stage of tumorigenesis when COX-2 expression is not yet induced

Microsomal PGE synthease-1 (mPGES-1) is an inducible PGE-converting enzyme that functionally couples with COX-2 for PGE_2 biosynthesis, and the expression of mPGES-1 is induced in cancer tissues together with that of COX-2 [19, 20]. Accordingly, the PGE_2 level is increased in cancer tissues due to the expression of both COX-2 and mPGES-1. Importantly, a disruption of the *Ptges* gene encoding mPGES-1 in $Apc^{\Delta 14}$ mice, another FAP model, and in a chemical carcinogen-treated colon cancer mouse model results in significant decreases in the PGE_2 level in the intestinal mucosa, which causes further suppression of intestinal tumor development [21, 22]. There are four G-protein coupled receptors for PGE_2, namely, EP1, EP2, EP3, and EP4. Signaling through EP2 and EP4 increases intracellular cyclic AMP and has been shown to be important for tumor development. Disruption of the *Ptger2* gene encoding EP2 in $Apc^{\Delta 716}$ mice causes significant suppression of intestinal polyp development, whereas disruption of *Ptger1* and *Ptger3* does not affect the tumor phenotype [23]. EP2 signaling accelerates angiogenesis in intestinal polyps through the induction of the vascular endothelial growth factor (VEGF) and basic fibroblast growth factor expression [24]. Moreover, EP2 signaling has been shown to activate Wnt signaling in colon cancer cells through the activation of the PI3K/Akt pathway and the direct association between G protein and Axin, thus resulting in the stabilization of β-catenin [25]. These results indicate that the COX-2/mPGES-1/PGE_2/EP2 pathway is important for tumor development through the involvement of several different mechanisms (Fig. 1). Although several studies have identified possible mechanisms underlying the COX-2/PGE_2 pathway in cancer development [8, 9], the key function of the COX-2/PGE_2 pathway in in vivo cancer development has not yet been fully elucidated.

The expression of proinflammatory cytokines, including TNF-α and IL-6, is induced in the inflammatory microenvironment of cancer, and mouse genetic studies have shown that these cytokines also promote tumorigenesis. TNF-α was originally recognized as a tumor necrosis factor; however, it is now established that TNF-α exhibits important tumor-promoting functions [26]. Inhibition of TNF-α signaling by disruption of TNF-α or its receptor gene results in the suppression of skin carcinogenesis and inflammation-associated colon cancer development [27–29]. TNF-α activates transcription factor NF-κB, which further induces the expression of inflammatory factors, including COX-2, IL-6, IL-8, and TNF-α itself, forming an inflammatory network in tumor microenvironment [30] (Fig. 2). Genetic inhibition of NF-κB in colitis-associated colon cancer model mice results in significant suppression of tumor development [31]. On the other hand, IL-6 expression is induced in colon cancer [32]. IL-6 signaling activates Stat3 through the gp130 receptor. Notably, disruption of IL-6 or Stat3 genes in colitis-associated colon cancer model mice results in significant suppression of colonic tumors [33, 34]. Taken together, these results indicate that the COX-2/PGE_2, TNF-α/NF-κB, and

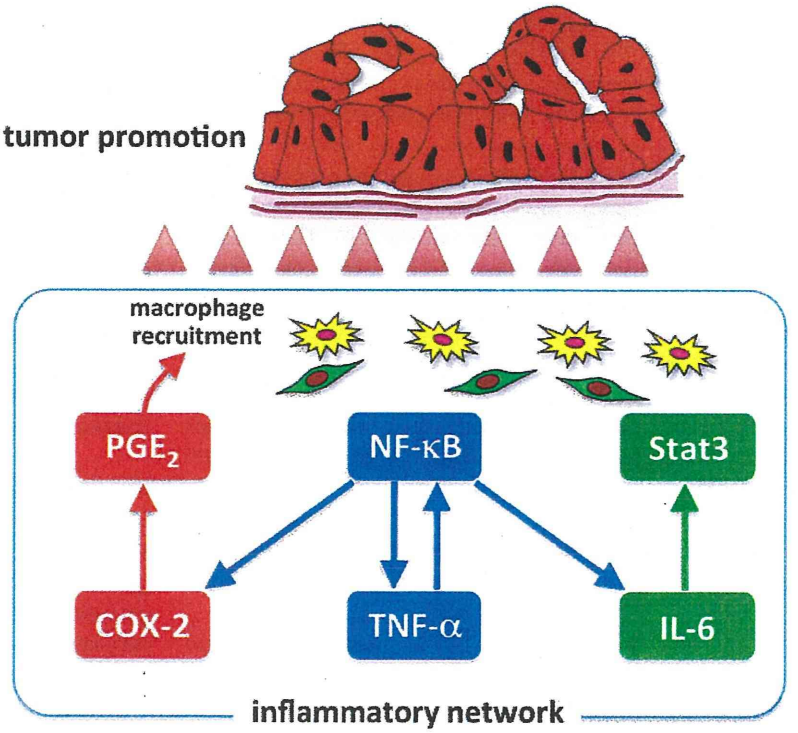

Fig. 2 Schematic diagram showing inflammatory network in tumor microenvironment. Induction of COX-2/PGE_2 pathway is responsible for the construction of an inflammatory microenvironment where macrophages are recruited. NF-κB is activated in tumor-associated macrophages, resulting in the induction of inflammatory factors including COX-2, TNF-α, and IL-6. TNF-α and IL-6 activate the transcription factors NF-κB and Stat3, respectively, forming an inflammatory network in the tumor microenvironment. Such network plays a critical role in tumor promotion

IL-6/Stat3 pathways construct the inflammatory tumor microenvironment required for the promotion of tumorigenesis [30] (Fig. 2).

Inflammation-associated gastric cancer mouse model

Preneoplastic changes induced by oncogenic Wnt signaling

Based on the results of the role of the COX-2/PGE_2 pathway in cancer development, we developed a PGE_2-associated gastritis and gastric cancer mouse models as follows: Canonical Wnt signaling (Wnt/β-catenin signaling) is important for the undifferentiated status of epithelial cells and thus induces tumorigenesis [35, 36]. Mutations in APC or β-catenin genes result in constitutive activation of canonical Wnt signaling, which causes tumor development in the entire intestinal tract [37, 38]. Nuclear or cytoplasmic accumulation of β-catenin, a hallmark of Wnt signaling activation, is detected in 30–50 % of gastric cancer tissues [39–42]. Moreover, mutations in the β-catenin gene have been found in a subpopulation of gastric cancers [39, 40, 43, 44]. These results indicate that Wnt signaling activation is an important oncogenic pathway for gastric cancer development.

To examine the role of Wnt activation in gastric tumorigenesis, we created *K19-Wnt1* transgenic mice that express Wnt1, a ligand for canonical Wnt signaling, using the *K19* gene promoter that is transcriptionally active in gastric epithelial cells [42]. Although *K19-Wnt1* mice do not develop gastric tumors, they do develop small aberrant cryptic foci on the surface of the gastric mucosa (Fig. 3a). Histologically, these lesions consist of dysplastic epithelial cells with increased cell proliferation and cytoplasmic β-catenin accumulation and are thus diagnosed as preneoplastic lesions [11, 12]. Importantly, macrophages infiltrate the stroma of preneoplastic lesions, while tissue macrophages are rarely detected in normal gastric mucosa (Fig. 3a). These results suggest that inflammatory microenvironments consisting of macrophages are constructed in the early stage of tumorigenesis and may be required for tumorous changes in Wnt-activated epithelial cells. To support this hypothesis, we demonstrated that macrophage-derived TNF-α promotes Wnt signaling activity in epithelial cells, which contributes to the development of preneoplastic lesions in *K19-Wnt1* mice [45].

Gastritis induced by the inflammatory COX-2/PGE_2 pathway

On the other hand, the COX-2/PGE_2 pathway is widely induced in gastric cancer, as found in colon cancer tissues [46]. *H. pylori* infection is tightly associated with gastric cancer, and the levels of the COX-2 expression and PGE_2 production are related to the status of *H. pylori* infection [47, 48]. Accordingly, it is possible that the induction of the COX-2/PGE_2 pathway is an important tumor-promoting mechanism of *H. pylori* infection. To examine the role of the COX-2/PGE_2 pathway in gastric tumorigenesis, we created another group of transgenic mice, *K19-C2mE* mice, that expresses *Ptgs2* and *Ptges* encoding COX-2 and mPGES-1, respectively, in the gastric mucosa [49]. *K19-C2mE* mice develop gastric hyperplasia and mucous cell metaplasia, which are histologically similar to spasmolytic polypeptide/trefoil factor 2 (TFF2)-expressing metaplasia (SPEM) (Fig. 3b) [49, 50]. SPEM is associated with human gastric cancers [51, 52], thus suggesting that SPEM is a possible precursor of gastric cancer [53]. SPEM development has also been observed to occur in the stomachs of several different gastric tumor model mice, including *Helicobacter*-infected mice, gastrin gene knockout mice, and gp130 receptor-active mutant mice [54–56], suggesting that the COX-2/PGE_2 pathway is responsible for SPEM formation in human gastric cancer as well as in these mouse models.

Hyperplastic lesions in *K19-C2mE* mice are associated with submucosal infiltration of lymphocytes, granulocytes, and mononuclear cells. Moreover, macrophage accumulation is also observed in the gastric mucosa (Fig. 3b). Notably, α-smooth muscle-expressing myofibroblasts are detected in *K19-C2mE* mouse inflamed gastric mucosa, and approximately 10 % of myofibroblasts is derived from bone marrow [57]. Myofibroblasts are also important components of the tumor microenvironment, together with tumor-associated macrophages. Accordingly, it is possible that induction of the COX-2/PGE_2 pathway is responsible for the construction of inflammatory microenvironment consisting of macrophages and myofibroblasts. Importantly, disruption of the TNF-α gene in *K19-C2mE* mice results in suppression of gastritis and SPEM development, while IL-1 receptor or Rag2 gene disruption does not affect these phenotypes [50]. Accordingly, it is possible that TNF-α induced in the COX-2/PGE_2-associated inflammatory microenvironment plays a key role in SPEM formation.

Gastric cancer induced by Wnt and the COX-2/PGE_2 pathway

By crossing *K19-Wnt1* and *K19-C2mE* transgenic mice, compound *K19-Wnt1/C2mE* transgenic mice were created that express *Wnt1*, *Ptgs2*, and *Ptges* simultaneously in the gastric epithelial cells, resulting in activation of Wnt signaling and the COX-2/PGE_2 pathway simultaneously in the stomach [42]. Importantly, *K19-Wnt1/C2mE* mice develop large gastric tumors at around 40 to 50 weeks of age with 100 % incidence (Fig. 3c). We thus named the *K19-Wnt1/*

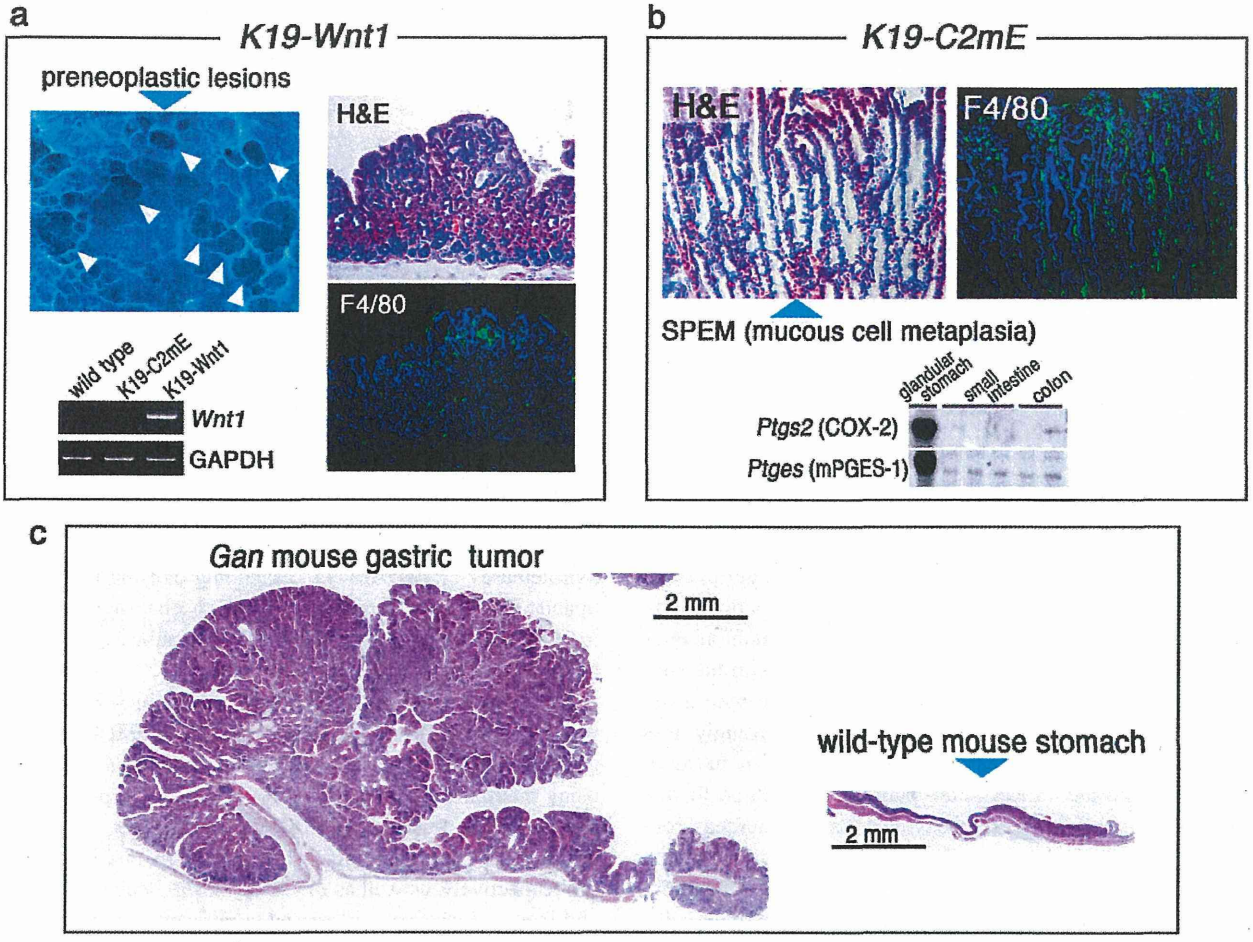

Fig. 3 Gastric phenotypes by Wnt activation, COX-2/PGE_2 induction, or both in combination. **a** *K19-Wnt1* mice expressing *Wnt1* in gastric epithelial cells develop preneoplastic lesions by Wnt activation (*left*, *right top*). Expression of *Wnt1* gene in *K19-Wnt1* mouse stomach was confirmed by RT-PCR (*left*, *bottom*). F4/80-positive macrophages are infiltrated in the stroma of preneoplastic lesion (*right*, *bottom*). **b** *K19-C2mE* mice express *Ptgs2* and *Ptges* in the stomach, resulting in the induction of the COX-2/PGE_2 pathway, and they develop SPEM lesion consisting of metaplastic mucous cells (*left*). F4/80-positive macrophages are accumulated in metaplastic and hyperplastic gastric mucosa (*right*). Expression of *Ptgs2* and *Ptges* in the glandular stomach was confirmed by Northern blotting (*bottom*). **c** *Gan* mice expressing *Wnt1*, *Ptgs2*, and *Ptges* in the stomach develop large gastric tumors (*left*) caused by the activation of both Wnt signaling and COX-2/PGE_2 pathway. Wild-type mouse stomach is shown as a control of the same magnification (*right*) (RT-PCR shown in **a** was reproduced from Oshima et al. [42] with permission from Elsevier, and Western blotting shown in **b** was reproduced from Oshima et al. [49] with permission from Nature Publishing)

C2mE mice as *Gan* mice, which stands for gastric neoplasia mice. Histologically, gastric tumors in *Gan* mice consist of dysplastic epithelial cells with an irregularly branching glandular structure, thus diagnosed as glandular-type gastric tumors. Moreover, the Ki-67 labeling index is significantly increased, indicating accelerated proliferation of tumor cells. Mucosal macrophage infiltration is also found in *Gan* mouse tumors, and the expression of proinflammatory cytokines and chemokines increases significantly. Accordingly, these results, taken together, indicate that Wnt signaling activation in the COX-2/PGE_2-associated inflammatory microenvironment cooperatively causes the development of gastric cancer. Importantly, the gene expression profiles of *Gan* mouse tumors are similar to those found in human intestinal-type gastric cancer [58], thus indicating that *Gan* mice recapitulate the development of human intestinal-type gastric cancer from genetic changes and host inflammatory responses to tumor morphology and gene expression profiles.

In the inflamed tissues, reactive oxygen species (ROS) and nitric oxide are induced, which play a role in tumor development. In the gastric tumor tissues of *Gan* mice, ROS level is also increased [59]. However, we have recently demonstrated that the expression of CD44 variant form (CD44v) is significantly induced in gastritis of *K19-C2mE* mice [60], and CD44v plays a critical role in tumor

development in *Gan* mice through protection of tumor cells from oxidative stress [59]. Accordingly, it is possible that ROS plays a role in tumorigenesis, and at the same time protection from ROS leads to tumor promotion.

Interestingly, macrophage infiltration and inflammatory responses in gastric tumors are significantly suppressed in germ-free *Gan* mice, although the COX-2/PGE_2 pathway is still activated [61]. Moreover, the gastric tumor volume decreases significantly in germ-free *Gan* mice. In contrast, *Helicobacter* infection in the stomachs of germ-free *Gan* mice induces inflammatory responses and the development of large gastric tumors. These results indicate that increased PGE_2 signaling is not sufficient to induce inflammatory responses in the stomach; however, bacterial infection together with the COX-2/PGE_2 pathway is required for the construction of the inflammatory microenvironment. It has been shown that Toll-like receptor (TLR) signaling is important for intestinal epithelial homeostasis, and the suppression of TLR signaling suppresses regenerative proliferation in injured mucosa [62]. Moreover, a disruption of the *Myd88* gene encoding MyD88, an important adaptor molecule of the TLR signaling pathway, results in the suppression of intestinal polyposis in Apc^{Min} mice with a decreased expression level of COX-2 [63]. Accordingly, it is possible that innate immune signaling activated by bacterial infection and the TLR/MyD88 pathway is required for the construction of the PGE_2-associated tumor microenvironment. On the other hand, it has been shown that epithelial cell-derived PGE_2 suppresses inflammatory cytokine expression in the LPS-stimulated bone marrow-derived cells, which is important for protection from bacterial infection-associated colitis [64]. It is therefore possible that PGE_2 has distinct functions on inflammatory cytokine expression in immune tolerance and in cancer microenvironment, although the mechanism underlying such difference remains to be investigated.

It is therefore expected that complicated interactions between tumor cells and the inflammatory microenvironment in human gastric cancer are repeated in *Gan* mouse gastric tumor tissues. Accordingly, using the *Gan* mouse model, we are able to uncover the mechanisms underlying PGE_2-associated inflammatory responses in gastric tumorigenesis. For example, we have determined that gastric tumor cells educate and activate stromal fibroblasts and bone marrow-derived cells to be myofibroblasts, resulting in the induction of the VEGF expression and enhancement of angiogenesis in tumor tissues [57]. Moreover, we examined the gene expression profiles of mRNAs and microRNAs (miRNAs) in *K19-C2mE* gastritis and *Gan* mouse gastric tumor tissues and found several novel mechanisms of inflammation in tumor development, as described in the following section.

Activation of epidermal growth factor receptor signaling by PGE_2-associated inflammation

EGFR signaling is an important target for cancer prevention [65]. The genetic or pharmacological inhibition of EGFR signaling in Apc^{Min} mice results in significant suppression of intestinal polyposis [66–68], indicating that EGFR signaling is required for tumor cell proliferation even when Wnt signaling is constitutively activated. Importantly, the combination treatment of Apc^{Min} mice with EGFR inhibitors and NSAIDs or COX-2 inhibitors suppresses intestinal polyposis more effectively than simple treatment, thus suggesting a possible link between the COX-2/PGE_2 pathway and EGFR signaling [67, 68]. It has already been shown using in vitro experiments that the PGE_2 pathway transactivates EGFR signaling through the activation of cSrc or MMPs [69–71] or the induction of EGFR ligands and a disintegrin and metalloproteinases 17 (ADAM17), a shedding enzyme for EGFR ligands [72–74]. We thus examined the mechanisms underlying EGFR activation by the COX-2/PGE_2 pathway in in vivo tumors using *K19-C2mE* and *Gan* mice.

We compared the expression levels of all EGFR ligands, their receptors, and shedding enzymes for EGFR ligands in gastritis and gastric tumors with those in normal stomachs using microarray results [58]. Surprisingly, the expression of most EGFR ligands, including *Areg*, *Ereg*, *Hbegf*, and *Btc*, encoding amphiregulin, epiregulin, HB-EGF, and betacellulin, respectively, as well as *Erbb2* and *Erbb3* encoding Her2 and Her3, respectively, increased significantly in both *K19-C2mE* mouse gastritis and *Gan* mouse gastric cancer compared with that observed in wild-type mice [75] (Fig. 4a). ADAM family proteases activate EGFR signaling by shedding the ectodomain of EGFR ligands to release them from the cell membrane, and some ADAM family genes are induced in a variety of cancer tissues, suggesting that ADAMs play a role in the activation of EGFR signaling in cancer development [76]. Notably, the expression of *Adam8*, *Adam9*, *Adam10*, and *Adam28* is increased significantly in the stomach in *K19-C2mE* gastritis and *Gan* mouse tumors. Taken together, these results indicate that the expression of EGFR ligand members, their receptors, and ADAM family members is upregulated simultaneously in the PGE_2-associated inflammatory microenvironment, which causes activation of EGFR signaling in cancer cells. Consistently, EGFR is phosphorylated in the epithelial cells of *K19-C2mE* and *Gan* mouse stomachs, indicating the activation of EGFR, which is not found in the wild-type mouse gastric mucosa (Fig. 4b). Among the four PGE_2 receptors, the expression of EP4 is significantly increased in the stomachs of both *K19-C2mE* and *Gan* mice [77]. Notably, treatment of *Gan* mice with EP4 receptor inhibitors results in significant decreases in the EGFR ligand and

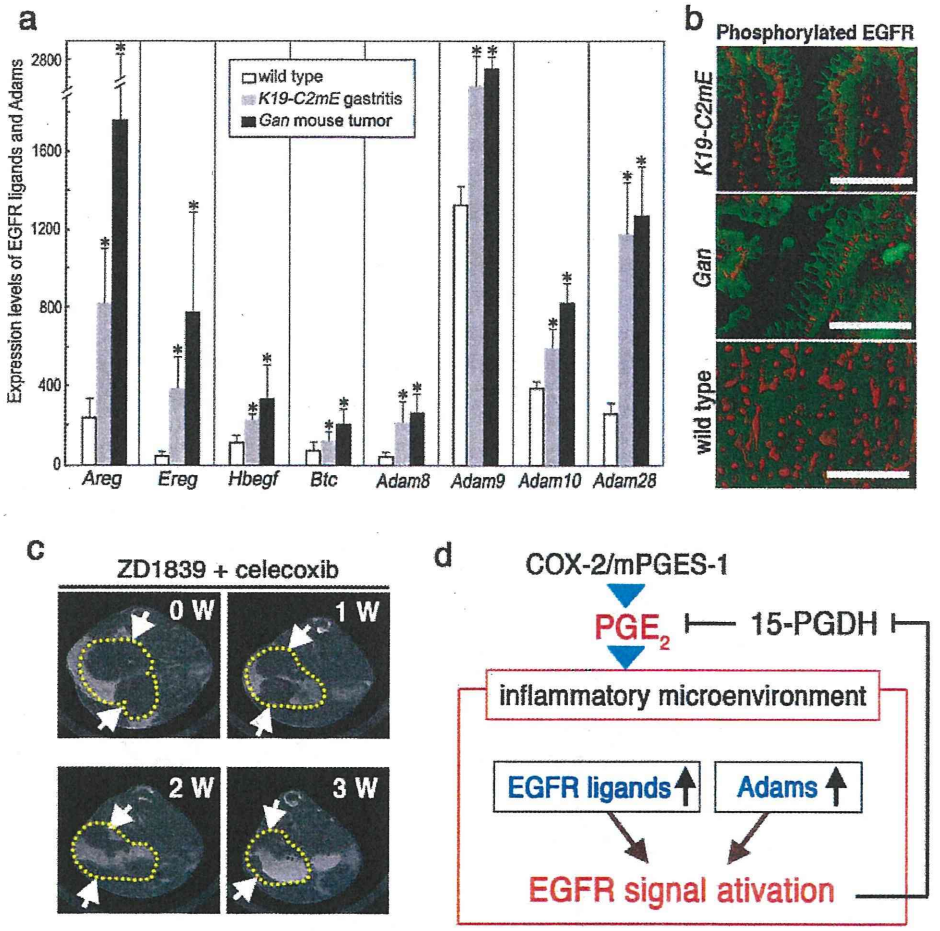

Fig. 4 Activation of EGFR signaling in COX-2/PGE_2 pathway-induced inflammatory microenvironment. **a** Expression of EGFR ligands and Adam family proteases is upregulated in both *K19-C2mE* mouse gastritis and *Gan* mouse gastric tumors. **b** Phosphorylated EGFR is detected by immunohistochemistry (*green*) in gastric epithelial cells of *K19-C2mE* mice and *Gan* mice, which is not found in the wild-type mouse stomach. Nuclei are stained with DAPI (*red*). **c** X ray CT photographs of a *Gan* mouse that was treated with EGFR inhibitor, ZD1839, and COX-2 inhibitor, celecoxib, in combination for 3 weeks. Note that the gastric tumor volume decreased dramatically by drug treatment. **d** Schematic diagram of EGFR activation in the COX-2/PGE_2 pathway-associated inflammatory microenvironment. Induction of EGFR ligands and Adams cooperatively activates EGFR signaling, which further activates PGE_2 signaling through downregulation of 15-PGDH by positive feedback mechanism (**a**, **b**, and **c** were reproduced from Oshima et al. [73] with permission from Wiley-Blackwell)

ADAM expression levels [75], thus indicating that PGE_2 signaling via the EP4 receptor is responsible for EGFR activation in gastric epithelial cells.

EGFR signaling activates the MAPK and PI3K/Akt pathways, which results in phosphorylation of Erk1/2 and Akt [78]. Notably, the phosphorylation levels of Erk1/2 and Akt in *Gan* mouse gastric tumors are decreased significantly by treatment of the mice with the COX-2 inhibitor celecoxib. Accordingly, it is possible that the COX-2/PGE_2 pathway is a major cause of activation of EGFR signaling in gastric cancer cells. On the other hand, EGFR signaling can activate the COX-2/PGE_2 pathway as follows: The cellular PGE_2 level is regulated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which catalyzes and thus inactivates prostaglandins. Importantly, the expression of 15-PGDH is downregulated by EGFR signaling [79], resulting in the maintenance of an increased PGE_2 level. Moreover, disruption of the 15-PGDH gene in Apc^{Min} mice accelerates intestinal tumorigenesis [80]. Accordingly, the COX-2/PGE_2 pathway activates EGFR signaling through the induction of EGFR ligands and ADAM proteases, which in turn downregulates 15-PGDH, resulting in an increased level of PGE_2 in the inflammatory microenvironment. Such PGE_2/EGFR signaling feedback loops therefore play an important role in gastric cancer development (Fig. 4d).

Treatment of *Gan* mice with either EGFR inhibitor, ZD1839, or celecoxib significantly reduces gastric tumor volume [75] (Fig. 4c). Importantly, combination treatment of *Gan* mice with ZD1839 and celecoxib results in the complete regression of gastric tumors, possibly through inhibition of individual pathways as well as feedback loops between PGE_2 and EGFR. These results suggest that the use of such combination treatment is an effective preventive strategy against gastric cancer development.

Downregulation of tumor suppressor microRNA, miR-7, by PGE_2-associated inflammation

miRNAs are single-strand, small, noncoding RNAs that regulate gene expression via post-transcriptional interference of target mRNAs [81, 82]. Accordingly, miRNAs can

function as oncogenes or tumor suppressors through the inhibition of tumor suppressor genes or of oncogene expression, respectively [83, 84]. Several mechanisms for dysregulation of the miRNA expression have been demonstrated, such as genetic or epigenetic alterations or transcriptional or post-transcriptional mechanisms [85]. Notably, it has also been shown that inflammation induces the expression of oncogenic miRNAs [86]. For example, miR-155 and miR-21 are oncogenic miRNAs that are induced by NF-κB, TLR, interferon-β, or the Stat3 pathway [87–89]. NF-κB and Stat3 are activated by TNF-α and IL-6, respectively, and are key factors in the network of tumor microenvironment [30] (Fig. 2).

Microarray analyses indicate that 50 microRNAs are upregulated (>2.0-fold) and 42 miRNAs are downregulated (<0.5-fold) in *Gan* mouse gastric tumors [90] (Fig. 5a). Among these miRNAs, 21 and 29 show the same upregulation and downregulation, respectively, in *K19-C2mE* mouse gastritis, thus indicating that expression changes in these miRNAs (21 up and 29 down) in gastric tumors are caused by COX-2/PGE_2 pathway-associated inflammatory responses. Notably, the oncogene miRNAs miR-21 and miR-155 [91] are upregulated both in *K19-C2mE* mouse gastritis and *Gan* gastric tumors, whereas the tumor suppressor miRNAs miR-145 and miR-7 [92, 93] are downregulated in both tissues. Accordingly, it is possible that

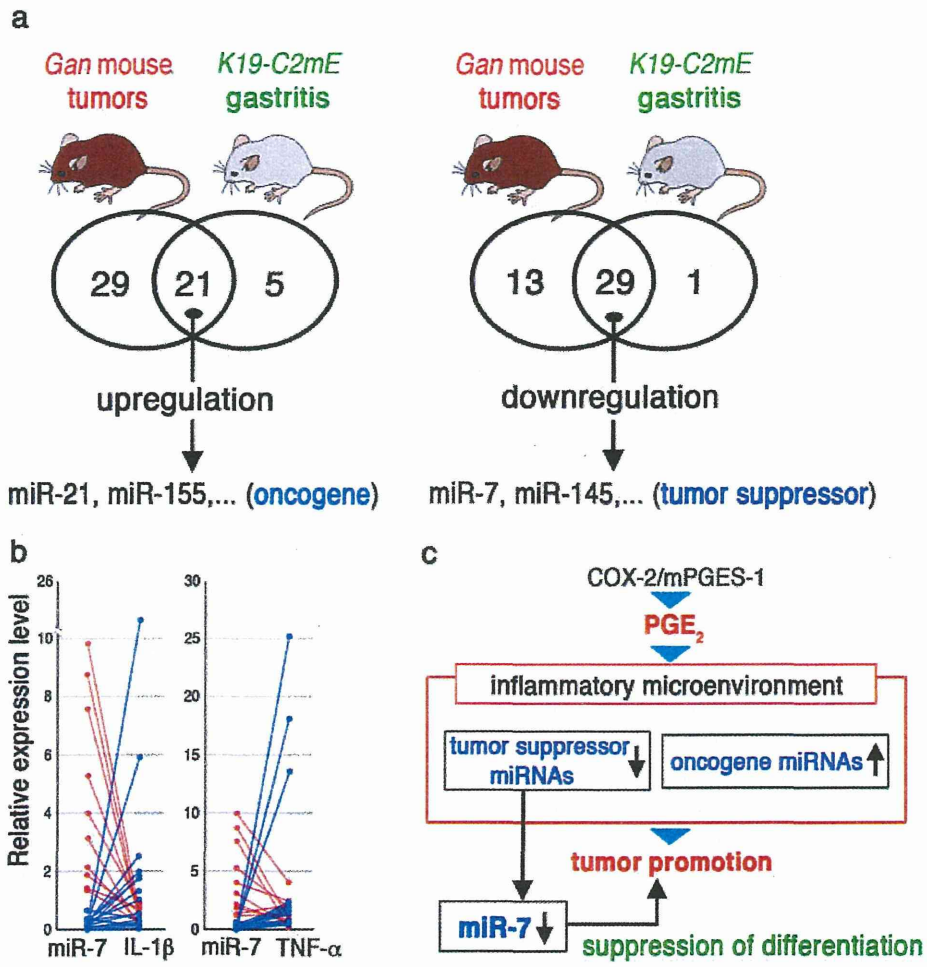

Fig. 5 Downregulation of tumor suppressor miRNA, miR-7, in COX-2/PGE_2 pathway-induced inflammatory microenvironment. **a** Venn diagrams of upregulated and downregulated miRNAs in *K19-C2mE* mouse gastritis and *Gan* mouse gastric tumors. Note that 21 and 29 miRNAs are upregulated and downregulated, respectively, both in gastritis and gastric tumors, indicating inflammation-dependent expression changes. Notably, oncogene miRNAs, miR-21 and miR-155, are upregulated, whereas tumor suppressor miRNAs, miR-7 and miR-145, are downregulated simultaneously both in gastritis and gastric tumors. **b** Inverse relations between the expression levels of miR-7 and inflammatory cytokines, TNF-α and IL-1β, in human gastric cancer tissues: namely, cytokine high gastric cancers show lower miR-7 level, indicating that the severity of inflammatory responses is associated with miR-7 downregulation. **c** Schematic diagram of miR-7 downregulation in PGE_2-associated inflammatory microenvironment. Downregulation of miR-7 promotes gastric tumorigenesis possibly through suppression of epithelial differentiation (**a** and **b** were reproduced from Kong et al. [88] with permission from Nature Publishing)