Microsomal PGE synthase-1 (mPGES-1) is an enzyme that converts PGH_2 to PGE_2, and expression of mPGES-1 is induced in gastric and colon cancers similar to COX-2 [42, 43]. Notably, disruption of *Ptges* gene encoding mPGES-1 in $Apc^{\Delta 14}$ mice or AOM-treated mice resulted in a marked decrease in the PGE_2 level in the intestinal mucosa, which led to a further significant suppression of intestinal tumorigenesis [44, 45]. Taken together, these results indicate that the simultaneous expression of COX-2 and mPGES-1 is required for intestinal tumorigenesis through the induction of PGE_2 signaling.

In gastric cancer tissues, induction of COX-2 is found in approximately 70% of cases [46, 47], and mPGES-1 expression is also induced [42], suggesting that the COX-2/PGE_2 pathway is also important for gastric tumorigenesis. In the *K19-C2mE* transgenic mice, an increased PGE_2 level causes inflammatory infiltration and metaplastic hyperplasia in the gastric mucosa [29]. Although Wnt activation alone is not sufficient for tumor development in *K19-Wnt1* mice, *Gan* mice (compound mutants of *K19-Wnt1* and *K19-C2mE* mice) develop inflammation-associated gastric tumors with 100% incidence [26], indicating that cooperation of the Wnt and PGE_2 pathways can lead to gastric tumorigenesis. Moreover, gastric inflammation and tumorigenesis were significantly suppressed in *Gan* mice when the mice were treated with a COX-2 inhibitor, celecoxib, or an EP4 receptor inhibitor [48, 49]. These results indicate that COX-2/PGE_2/EP4-induced inflammation is involved in the development of gastric cancer. Signaling through both EP2 and EP4 stimulates the intracellular cyclic AMP signaling pathway. It is therefore possible that either EP2 or EP4 receptor signaling plays an important role in gastrointestinal tumorigenesis (Fig. 1).

The Toll-like receptor (TLR)/MyD88 pathway for COX-2 induction in gastrointestinal tumors

Expression of COX-2 and mPGES-1 is detected predominantly in stromal cells, including macrophages and fibroblasts, but not in the epithelial cells of mouse intestinal polyps, and the same is true for human colon polyps [36, 50–52]. Heterozygous mutations in the *Lkb1*, *Smad4*, or *Cdx2* gene lead to the development of gastrointestinal hamartomas, which show histological characteristics distinct from those of dysplastic adenomas developed in *Apc* mutant mice [52–55]. Notably, expression of COX-2 and mPGES-1 is detected in the stromal cells, but not in tumor epithelial cells, in these models [56], thus indicating that the COX-2/PGE_2 pathway is induced in the tumor stroma by a common mechanism, regardless of the type of tumor.

Several studies have suggested the commensal flora to play a role in the homeostasis of the intestinal mucosa. Toll-like receptors (TLRs) are a family of pattern-recognition receptors that detect the molecular products of microorganisms. MyD88 is an adaptor molecule for the TLR-mediated induction of inflammatory cytokines. It has been shown that a disruption of *Tlr2/4* or *Myd88* genes encoding TLR2/4 or MyD88, respectively, in mice results in the impaired mucosal repair of DSS-induced ulcers [57], suggesting that infectious stimulation through the TLR/MyD88 pathway is important for the regeneration process of injured mucosa. Moreover, stromal macrophages are also required for the mucosal repair of DSS-induced ulcers in the colon [58]. Accordingly, it is possible that stromal macrophages are activated by the TLR/MyD88 pathway. In the case of intestinal tumorigenesis, cancer cells may use such a TLR/MyD88-induced regeneration system to increase their proliferation.

The treatment of mice with DSS induces COX-2 expression and PGE_2 production, predominantly in macrophages of the inflamed colon mucosa. However, such COX-2 induction is not found in the mice lacking the TLR4/MyD88 pathway [59], thus indicating the role of bacterial infection in inducing COX-2 expression in colitis tissues. Importantly, AOM/DSS treatment-induced colon tumor development was dramatically suppressed in *Tlr4−/−* mice [60], while exogenous administration of PGE_2 promoted CAC development in the AOM/DSS-treated *Tlr4−/−* mice [61]. Moreover, bone marrow chimera experiments have indicated that TLR4 expression in the intestinal epithelial cells, but not in myeloid cells, is required for CAC development [62]. Taken together, these results suggest that bacterial infection stimulates the TLR/MyD88 pathway in epithelial cells, which leads to activation of stromal macrophages, thus resulting in the induction of the COX-2/PGE_2 pathway in the tumor stroma (Fig. 2).

In contrast to the AOM/DSS model, intestinal polyposis in the *Apc* mutant mice is not associated with UC. It is therefore possible that COX-2 expression is induced by a different mechanism in sporadic colon cancer compared to that in IBD-related tumors. However, Apc^{Min} *Myd88−/−* mice showed significant suppression of intestinal polyposis with dramatically decreased mortality compared with control Apc^{Min} *Myd88+/+* mice [63, 64]. Moreover, the induction of COX-2 expression was also suppressed in Apc^{Min} *Myd88−/−* mouse intestinal tumors [63]. Bone marrow chimera experiments also indicated that MyD88 expression in the epithelial cells was important for intestinal tumorigenesis [64]. These results have demonstrated that activation of the TLR/MyD88 pathway in epithelial cells is also important for COX-2 expression in non-IBD-related colon cancer (Fig. 2).

If bacterial infection is also required for COX-2 induction in the stomach, a low bacterial count due to the acidic environment in the stomach may protect against COX-2 expression in tumorous lesions. Therefore, it is possible that COX-2 induction through the TLR/MyD88 pathway is one of the important mechanisms by which *Helicobacter pylori* infection promotes gastric cancer.

It has also been shown that TLRs can be stimulated with endogenous ligands, including heat shock proteins and various products of the extracellular matrix [65]. Accordingly, it is possible that tumor cell proliferation causes tissue damage, releasing endogenous ligands for TLRs, thus resulting in their activation, which induces COX-2 expression in stromal macrophages. Such "cancer-induced inflammation" may also be one of the mechanisms responsible for the generation of an inflammatory microenvironment, especially in cancers that are not associated with infection [65].

The paradox of the COX-2 pathway in colitis-associated cancer

Although COX-2 inhibition causes suppression of gastrointestinal tumorigenesis, treatment with NSAIDs or a COX-2 inhibitor exacerbates DSS-induced colon injury in rodent models [66]. Consistently, *Ptgs2* gene knockout mice exposed to DSS showed exacerbated phenotypes, such as more severe inflammation, compared with wild-type mice [67]. One of the functions of PGE_2 is to protect the gastrointestinal mucosa. Therefore, COX-2 is important for mucosal protection and regeneration in the DSS-treated mouse colon. Notably, treatment of *Ptgs2* knockout mice with AOM/DSS resulted in a significant increase in tumors, with severe inflammatory responses [68, 69], which appears to be contradictory to the results showing that *Ptgs2* gene disruption causes significant suppression of intestinal polyposis in *Apc* mutant mice [34, 35]. It is possible that in the case of severe UC, cytokine signaling is highly activated and may be sufficient for tumor promotion if the COX-2/PGE_2 pathway is blocked (Fig. 2).

The TNF-α/NF-κB pathway in gastrointestinal tumorigenesis

TNF-α is one of key regulators of inflammatory responses. Although TNF was originally recognized as a tumor-necrotizing factor, accumulating evidence has indicated that TNF-α has tumor-promoting functions [70]. TNF-α signaling activates NF-κB, which further induces the expression of inflammatory factors including COX-2, IL-6, IL-8, and TNF-α itself (Fig. 1). Several genetic studies have demonstrated a link between the TNF-α/NF-κB pathway and cancer development [71].

Conditional disruption of *Ikbkb* gene encoding IKKβ in myeloid cells, which results in specific inhibition of NF-κB, caused significant suppression of the tumor incidence in AOM/DSS-treated mice, and was associated with decreased expression of cytokines and COX-2 [72]. Conditional deletion of *Ikbkb* in epithelial cells also suppressed AOM/DSS-induced tumorigenesis [72]. Similar results were found in TNF-α receptor gene knockout mice [73]. AOM/DSS treatment in mice lacking *Tnfrsf1a* gene encoding TNF receptor p55 (TNF-Rp55) resulted in attenuated tumor formation, with reduced inflammatory cell infiltration compared with findings in wild-type mice. Moreover, wild-type mice transplanted with *Tnfrsf1a*-deficient bone marrow developed significantly fewer tumors after AOM/DSS treatment [73], indicating that TNF-α stimulation of myeloid cells is important for tumorigenesis. Accordingly, it is possible that the TNF-α-induced NF-κB activation in myeloid cells is important for CAC development, and NF-κB activation in epithelial cells also contributes to tumor formation (Fig. 2).

It has also been shown that a disruption of *Ccr2* gene encoding a CCL2-specific receptor, CCR2, led to significantly decreased macrophage infiltration and lower tumor numbers when mice were treated with AOM/DSS [74]. CCL2 is a chemokine that is chemotactic for monocytes and macrophages [75]. Taken together, these findings indicate that the activation of NF-κB in activated macrophages by TNF-α in an autocrine or paracrine manner is important for the promotion of intestinal tumorigenesis (Fig. 2). The inhibition of TNF-α in *Apc* mutant mice by treatment with an anti-TNF-α antibody also suppressed intestinal polyposis, with the suppression of angiogenesis [76]. Accordingly, it is possible that TNF-α/NF-κB activation is important in both IBD-related and sporadic colon carcinogenesis.

An important role for the TNF-α/NF-κB pathway was also discovered in *Mdr2* knockout mice that develop inflammation-associated hepatocellular carcinoma (HCC) [77]. In this mouse model, NF-κB is activated in the liver by TNF-α signaling, and the inhibition of NF-κB significantly suppressed the development of HCC after 7 months of age [77]. On the other hand, preneoplastic dysplastic lesions in younger mice were not affected by NF-κB inhibition. It is possible that the TNF-α/NF-κB pathway is not required for the initiation step, but it does play a role in the promotion step of HCC development.

The role of the TNF-α/NF-κB pathway in gastric tumorigenesis in *Gan* mice has not yet been examined. However, *Tnf−/− K19-C2mE* mice showed significant suppression of gastritis and hyperplasia compared with control *K19-C2mE* mice, although the COX-2/PGE_2

pathway was still activated by the transgenic expression of COX-2 and mPGES-1 [78]. It is thus conceivable that the TNF-α/NF-κB pathway is activated in PGE_2-associated gastritis, and that this contributes to inflammation-associated gastric tumorigenesis.

The IL-6/gp130/Stat3 pathway in gastrointestinal tumorigenesis

One of the NF-κB-inducible cytokines is IL-6, which is important for immune responses, cell survival, apoptosis, and proliferation [79]. The expression of IL-6 is often upregulated in tumor tissues and in the sera of humans and mice with cancers, including colon cancer [80]. The IL-6 cytokine family signals through a common receptor, gp130, which activates Stat3 (Fig. 1). Stat3 plays an important role in the development of a variety of cancers, including CAC [81–84]. AOM/DSS-induced CAC development was significantly suppressed in conditional *Stat3*-knockout mice that lacked *Stat3* in the intestinal epithelial cells and also in *Il6−/−* mice [81, 82]. Survival and proliferation of tumor cells were suppressed in these mutant mice. On the other hand, the number and size of AOM/DSS-induced colon tumors increased significantly in $gp130^{757F/F}$ mice, in which gp130-dependent Stat signaling is constitutively activated [81]. These results indicate that Stat3 activated in epithelial cells plays an important role in the promotion of intestinal tumorigenesis (Fig. 2).

It has been shown that $gp130^{757F/F}$ mice develop gastric tumors with abundant infiltration of inflammatory cells [85]. Moreover, heterozygous mutations of the Stat3 gene in $gp130^{757F/F}$ mice reduced the incidence and multiplicity of gastric tumors, with the suppression of inflammatory responses [86, 87]. These results indicate that Stat3 is also an important tumor-promoting factor in gastric tumorigenesis, and that it is activated by the inflammatory network of the tumor microenvironment. Transforming growth factor (TGF)-β signaling promotes epithelial differentiation, and thus, suppression of the TGF-β signaling pathway has been thought to promote gastrointestinal tumorigenesis. Notably, the activation of Stat3 in $gp130^{757F/F}$ cells desensitizes them to TGF-β by inducing inhibitory Smad7. This may be one of the mechanisms by which Stat3 promotes tumor formation [86]. IL-11 is another member of the IL-6 cytokine family and also signals through gp130. Interestingly, disruption of the IL-11 co-receptor in $130^{757F/F}$ mice significantly ablated gastric tumorigenesis [88]. Because IL-11 is upregulated in human and mouse gastric tumors, these results suggest that the IL-11/Stat3 pathway, together with IL-6/Stat3, promotes gastric tumorigenesis.

Inflammatory cells that promote or suppress tumorigenesis

The major source of inflammatory cytokines and prostaglandins in tumor tissues is macrophages. Tumor-associated macrophages (TAMs) have been shown to promote the progression and metastasis of cancer [89]. TAMs can be classified into several distinct groups by their functions, such as inducing inflammation, invasion, angiogenesis, or metastasis. Macrophages are polarized to either the classical M1 type or the alternative M2 type, and it has been suggested that TAMs are polarized to M2 or M2-like types [90]. It has been shown that CD4+ T cells regulate the polarization of macrophages to the M2 type in mammary tumors [91]. It has recently been shown that COX-2 is important for the M2-polarization of TAMs in Apc^{Min} mouse tumors [92]. Accordingly, it is possible that the COX-2/PGE_2 pathway-induced inflammatory network is important for the education of macrophages into the protumorigenic M2 or M2-like types. Importantly, depletion of functional macrophages in $Apc^{\Delta 716}$ mice by crossing them with op/op mutant mice resulted in significant suppression of intestinal polyposis [93]. Moreover, inhibition of macrophage recruitment in AOM/DSS-treated mice by *Ccl2* gene disruption resulted in the suppression of colon tumor development [74]. Accordingly, macrophages play an important role in both sporadic and IBD-related intestinal tumorigenesis (Fig. 2). In *Gan* mouse gastric tumors, macrophage depletion caused atrophic changes of the tumor cells and apoptosis of stromal cells [49]. Accordingly, it is possible that macrophages play a role in the maintenance of both tumor epithelial cells and stromal cells.

In the $Apc^{\Delta 468}$ mouse intestinal tumors, mast cells are preferentially enriched in the polyp tissues, and these mast cells express TNF-α [76]. Notably, the depletion of mast cells in $Apc^{\Delta 468}$ mice by bone marrow transplantation from $Kit^{Wsh/Wsh}$ mice caused significant suppression of intestinal polyposis, with a decreased level of TNF-α expression. It is therefore possible that both macrophages and mast cells are important components of the inflammatory network in the tumor microenvironment.

Bone marrow transplantation from *Rag2−/−* mice did not affect the intestinal tumorigenesis in $Apc^{\Delta 468}$ mice [76]. Consistently, the gastritis phenotype was not altered in the *K19-C2mE Rag2−/−* mouse stomach [78]. These results indicate that lymphocytes are not required for the construction of the inflammatory network and the promotion of tumorigenesis in the gastrointestinal tract. Importantly, however, the adaptive transfer of $CD4^+$ $CD25^+$ regulatory T cells to Apc^{Min} mice dramatically reduced the number of intestinal polyps, with the induction of necrosis of tumor cells [94, 95]. Moreover, such regression of intestinal

polyps was not found when $CD4^+$ $CD25^+$ cells were prepared from *Il10−/−* mice.

The transfer of $CD4^+$ $CD25^+$ T cells to *Helicobacter hepaticus*-infected *Rag2−/−* mice, another IBD-related colon cancer model, resulted in suppression of colitis and tumor development, but *Il10*-disrupted $CD4^+$ $CD25^+$ T cells could not suppress the development of CAC [23, 24]. IL-10 suppresses inflammatory responses, thus indicating that regulatory T cells expressing IL-10 suppress intestinal tumorigenesis by inhibiting the formation of the inflammatory network. Although $CD25^+$ $Foxp3^+$ T cells are found in Apc^{Min} mouse polyp stroma, they no longer express IL-10, and instead switch to the production of IL-17 [95]. It is therefore possible that anti-inflammatory regulatory T cells ($Foxp3^+$ IL-10^+ IL-17^-) shift to pro-inflammatory T cells ($Foxp3^+$ IL-10^- IL-17^+) in polyp tissues [96]. Moreover, the ablation of *Il17a* gene in Apc^{Min} mice significantly suppressed the development of intestinal polyps and inflammatory cytokine expression, indicating that T-cell-derived IL-17 plays an important role in intestinal tumorigenesis [97].

Concluding remarks

The development of the inflammatory network in tumor tissues and its possible roles are summarized in Fig. 2. Chronic infection or endogenous ligands derived from tumor cells stimulate the TLRs of epithelial cells, leading to the activation of MyD88. The activation of the epithelial TLR/MyD88 pathway further induces COX-2 expression and PGE_2 production in stromal macrophages through the TNF-α/NF-κB pathway. Epidemiological studies and genetic experiments have demonstrated that COX-2 and its downstream product, PGE_2, play an important role in gastrointestinal tumorigenesis. NF-κB is activated by TNF-α in TAMs, which further induces the expression of TNF-α, IL-6, and COX-2. TNF-α in turn stimulates both tumor epithelial cells and stromal cells, activating NF-κB in these cells, which promotes tumorigenesis. On the other hand, IL-6 activates Stat3 through gp130 in epithelial cells, thus leading to an increase in cell cycling and a decrease of apoptosis. The induction of the COX-2/PGE_2 pathway is important for the development of such an inflammatory tumor microenvironment. When the TNF-α/NF-κB and/or IL-6/Stat3 pathways are activated beyond a threshold, they can promote tumorigenesis if the COX-2/PGE_2 pathway is inhibited. In the inflammatory microenvironment, not only TAMs, but also mast cells and IL-17-expressing T cells, infiltrate and contribute to tumor development. Therefore, targeting the inflammatory network in tumor tissues by the inhibition of PGE_2, NF-κB, Stat3, or downstream pathways may provide an effective preventive or therapeutic strategy against gastrointestinal cancer.

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Conflict of interest None.

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Genetic reconstitution of tumorigenesis in primary intestinal cells

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Animal models for human colorectal cancer recapitulate multistep carcinogenesis that is typically initiated by activation of the Wnt pathway. Although potential roles of both genetic and environmental modifiers have been extensively investigated in vivo, it remains elusive whether epithelial cells definitely require interaction with stromal cells or microflora for tumor development. Here we show that tumor development could be simply induced independently of intestinal microenvironment, even with WT murine primary intestinal cells alone. We developed an efficient method for lentiviral transduction of intestinal organoids in 3D culture. Despite seemingly antiproliferative effects by knockdown of adenomatous polyposis coli (*APC*), we managed to reproducibly induce *APC*-inactivated intestinal organoids. As predicted, these organoids were constitutively active in the Wnt signaling pathway and proved tumorigenic when injected into nude mice, yielding highly proliferative tubular epithelial glands accompanied by prominent stromal tissue. Consistent with cellular transformation, tumor-derived epithelial cells acquired sphere formation potential, gave rise to secondary tumors on retransplantation, and highly expressed cancer stem cell markers. Inactivation of *p53* or phosphatase and tensin homolog deleted from chromosome 10, or activation of *Kras*, promoted tumor development only in the context of *APC* suppression, consistent with earlier genetic studies. These findings clearly indicated that genetic cooperation for intestinal tumorigenesis could be essentially recapitulated in intestinal organoids without generating gene-modified mice. Taken together, this in vitro model for colon cancer described herein could potentially provide unique opportunities for carcinogenesis studies by serving as a substitute or complement to the currently standard approaches.

colon carcinogenesis | shRNA | primary culture | Matrigel | validation

Accumulation of multiple genetic alterations underlies colon carcinogenesis, in which inactivation of adenomatous polyposis coli (*APC*) is an initiating event leading to the development of adenoma in most sporadic cases (1). Both *APC* inactivation and an activating mutation in the *CTNNB1* gene encoding β-catenin result in β-catenin accumulation through inhibition of its degradation, leading to constitutive activation of the Wnt pathway that is transcriptionally regulated by the β-catenin/transcription factor 4 (TCF) complex (2).

Widely used animal models for colorectal cancer (CRC) recapitulate tumor development in a similar manner. One is a mouse genetic model with a mutant allele of *APC*. Typically, multiple adenomas spontaneously develop predominantly in the small intestine through inactivation of the remaining allele (3, 4). The other is a chemically induced carcinogenesis model. Administration of azoxymethane (AOM) or a dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP), recapitulates colon carcinogenesis in rodents by introducing an activating mutation in *CTNNB1* (5, 6) or inactivating *APC*, by mutation (7) or post-transcriptional down-regulation by overexpressed staphylococcal nuclease and tudor domain containing 1 (*SND1*) (8). Potential roles of genetic or environmental factors have been extensively investigated with these models. For instance, disruption of *p53* (9, 10) or phosphatase and tensin homolog deleted from chromosome 10 (*PTEN*) (11, 12) or induction of oncogenic *Kras* (13–15) significantly promoted intestinal tumorigenesis only in the context of *APC* loss. Protumorigenic effects by active inflammation have been demonstrated by inducing colitis with dextran sodium sulfate (DSS) (16). Conversely, critical roles of microflora and basal inflammation underlying tumorigenesis were also demonstrated by genetic ablation of *Myd88* (17) and *STAT3* (18), key genes in the innate immunity and inflammation, respectively.

Recent genomic and expression profile analyses have revealed a huge number of genes with mutation, deletion, or aberrant expression in human CRC (19, 20). Forward genetic screens in mice have also identified a number of genes potentially involved in intestinal tumorigenesis (21). Candidate genes for CRC have been usually validated through generation of gene-modified mice. However, it might be unrealistic to take this approach for very many genes, given the amount of time and work required for the analysis of each gene. This situation is especially true if generation of conditional KO mice and intercrossing between multiple strains becomes necessary. Alternatively, functional analyses of the genes have been widely conducted in colon cancer cell lines and fibroblasts to investigate the relevance in tumor progression and to determine oncogenic potential, respectively. However, the results might not be directly extrapolated to early stages of intestinal tumorigenesis, underscoring the definite requirement for simple validation methods in normal intestinal cells.

Given that intestinal stem cells efficiently give rise to adenoma on activation of the Wnt pathway in vivo (22, 23), we postulated that a similar approach might induce tumor development in vitro, although the requirements of intestinal microenvironment remained elusive. With recent advances in long-term culture of intestinal stem cells (24), we set out to suppress *APC* in intestinal organoids with a lentivirus. We generated tumors from intestinal organoids, independently of the in vivo setting and without using gene-modified mice. Representative genetic cooperation for tumorigenesis could be recapitulated by taking this approach, likely establishing an in vitro model for CRC.

Results

Lentivirus-Based Efficient and Stable Gene Delivery to Intestinal Organoids. To reconstitute tumorigenesis in vitro, stem cells need to be stably transduced. We adopted lentiviral gene delivery for its high infection efficiency to primary cells, including quiescent stem cells (25). However, it was revealed that Matrigel inhibited viral transduction of intestinal epithelial cells (IECs) in 3D culture, despite its definite requirement for survival. To satisfy both the presence of Matrigel and accessibility to lentiviral

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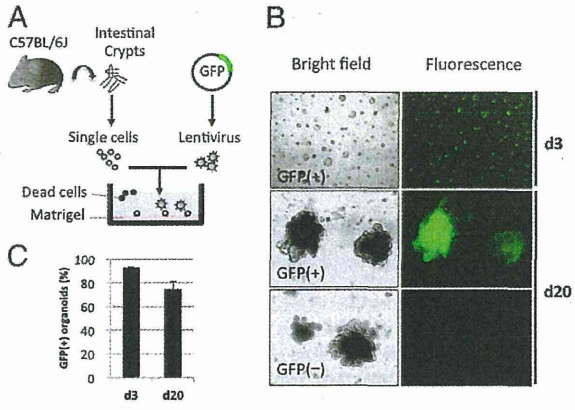

Fig. 1. Stable gene transduction of IECs in 3D culture. (*A*) Schematic diagram for lentiviral infection. Intestinal crypts isolated from C57BL/6J mice were dissociated into single cells and incubated with lentiviral particles encoding GFP for 16 h on Matrigel. (*B*) Stable and efficient transduction of organoids. Transduced organoids at day 3, at 40× magnification (*Top*). At day 20, transduced organoids consisting of GFP-positive cells (*Middle*). A non-GFP vector gave rise to only faint auto-fluorescence by dead cells at 100× magnification (*Bottom*). Representative images are shown. (*C*) Transduction efficiency to intestinal cells. Rate for GFP-positive organoids without drug selection is shown. GFP-positive and -negative organoids were counted under a microscope 48 h after the infection (day 3) or second subculture (day 20). Mean ± SD (n = 3) is shown.

particles, we coincubated dissociated single cells and viral particles on Matrigel (Fig. 1*A*), which achieved high transduction efficiency (Fig. 1*B*). About half of the cells composing organoids were viable, and ~20% of them attached to Matrigel with or without viral particles (Fig. S1), whereas no dead cells were observed on Matrigel. Attached cells readily developed into tiny circular organoids at day 3 (Fig. 1*B*), implying this procedure might enable preferentially capture intestinal cells of a highly proliferative nature. Even without drug selection, the GFP-positive rate was as high as 93% at day 3, which fell to 75% at day 20 (Fig. 1*C*), presumably due to slightly adverse effects by viral integration. Many organoids consisted of only GFP-positive cells at day 20, even after two rounds of subculture (Fig. 1*B*). Given the rapid turnover rate of IECs (24), we reasoned that organoids were likely reconstituted by stably transduced stem cells.

Wnt Pathway Activation in Organoids Transduced with Multiple Clones of shRNA Against *APC*. With this efficient technique, we introduced a total of five clones of potent shRNA against *APC* (shAPC) (Fig. S2*A*) individually into organoids. With a routine schedule for 3D culture (Fig. S2*B*), however, we frequently failed in propagation for any shAPC clone tested, even under drug selection (Fig. S2*C*), suggesting adverse effects by *APC* knockdown in vitro. In contrast, introduction of potent shp53 or shPTEN (Fig. S2*D*) resulted in steady propagation of organoids (Fig. 2*A*), which spontaneously became puro-resistant, suggesting a growth advantage of inactivating *p53* or *PTEN*. We later found that cointroducing all of the five shAPC clones together (hereafter referred to as shAPCs) reproducibly gave rise to rounded cystic organoids, which dominated the population over time (Fig. 2*A*; Fig. S2*C*). Similar structures have been previously documented for organoids from *APC*-deficient adenoma (26, 27), suggesting a link between the morphology and *APC* loss. However, we assumed that this might not be necessarily the case, because we knew that cystic shape could be induced independent of *APC* knockdown (e.g., under stressed culture conditions including freeze/thaw, drug selection, or too stringent dissociation), which prompted us to characterize the cystic organoids with shAPCs in more detail. We found that they were puro-resistant and indeed suppressed for expression of *APC* (Fig. S2*D*). In thin sections, they lost physiological properties such as polarity (Fig. 2*B*), differentiation (Fig. 2*C*), and cellular turnover (Fig. 2 *B* and *C*), consistent with perturbed differentiation and migration associated with *APC* inactivation (28). In addition, β-catenin accumulation indicative of Wnt pathway activation was evident (Fig. 2*D*), which was also confirmed by qPCR analysis demonstrating up-regulation of *Axin2* (Fig. 2*E*), a specific target of the β-catenin/TCF complex (29). These observations implied that the organoids with shAPCs might be essentially similar, if not identical, to those derived from *APC*-deficient adenoma.

Induction of Tumors from Organoids by RNAi-Mediated Suppression of *APC*. We next investigated whether suppression of *APC* in organoids could also lead to tumor development, as observed in adenoma in vivo. After 4 wk of culture, organoids with shAPCs corresponding to 5×10^5 cells were mixed with Matrigel and injected into nude mice. At 6 wk after injection, round and solid flesh-colored nodules frequently developed (Fig. 3*A*). They were characterized by epithelial glands and prominently infiltrated stromal cells (Fig. 3*B*). Active proliferation of epithelia was verified by high Ki-67 labeling index and inferred from β-catenin accumulation (Fig. 3*C*). Based on these features common to intestinal tumors, we classified them as "tumors." In contrast, organoids with the vector control gave rise to no nodules at all or small flat nodules with a gelatinous appearance, if any (Fig. 3*A*). As they histologically lacked epithelial glands (Fig. 3*B*), we classified them as "Matrigel plugs." In the absence of shAPCs, no tumor was induced by shp53 and/or shPTEN or from *p53*- and *PTEN*-deficient organoids (Fig. S3; Table 1), in line with earlier studies in vivo (11, 12, 30, 31). In some cases, organoids with

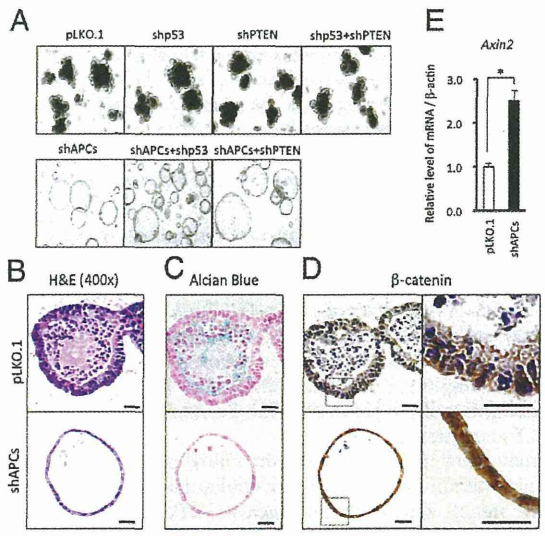

Fig. 2. RNAi-mediated suppression of *APC* in intestinal organoids. (*A*) Organoids transduced with various shRNA(s). Representative images at 4 wk after transduction are shown. Large rounded cysts were induced exclusively in the presence of shAPCs. pLKO.1 is an empty vector. (*B–D*) Transduced organoids in thin section. Serial sections were stained with H&E (*B*), Alcian blue (*C*), and β-catenin antibody (*D*). In organoids with shAPCs, intraluminal debris due to physiological turnover of intestinal cells is lost. Paneth cells stained red (*B*) and mucus stained blue (*C*) also became absent. Localization of β-catenin shifted from membrane to cytoplasm and nucleus (*D*). *Insets* in the left panel are enlarged in the right panel. (Scale bar, 20 μm.) (*E*) qPCR analysis of *Axin2* in transduced organoids. Relative expression level of mRNA to *β-actin* is shown. Mean ± SD (n = 3) is shown; $^*P < 0.01$.

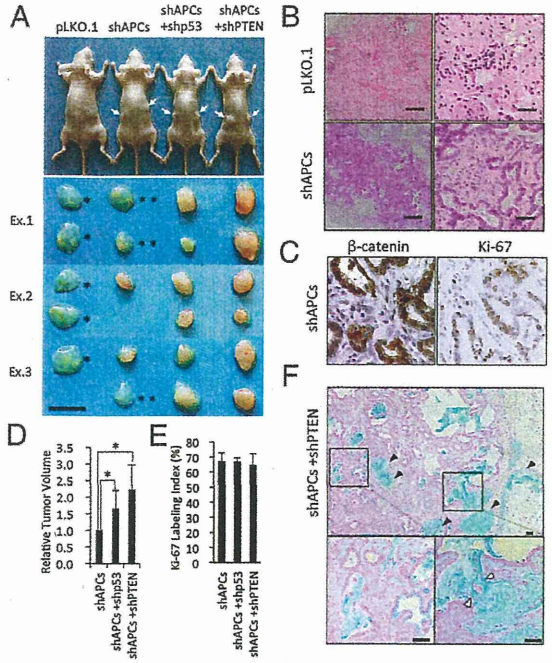

Fig. 3. RNAi-mediated induction of tumors from WT organoids. (*A*) s.c. tumors developed from injected organoids. Palpable nodules in nude mice (arrow) at 6 wk after injection (*Upper*). Excised nodules in three representative experiments (Ex.1–3). Matrigel plugs (asterisk), nontumor (double asterisks), or tumors (no asterisk) (*Lower*). (Scale bar, 10 mm.) (*B*) Histological features of the nodules. H&E staining of Matrigel plugs (*Upper*) and tumors with shAPCs alone (*Lower*) at 20× (*Left*) and 200× (*Right*) magnification. (Scale bar, 500 and 50 μm, respectively.) (*C*) Immunohistochemical analyses. Tumors with shAPCs alone stained for β-catenin (*Left*) and Ki-67 (*Right*). (Scale bar, 25 μm.) (*D*) Relative ratio of tumor volume. Mean ± SD ($n = 5$) is shown; $^*P < 0.05$. (*E*) Ki-67 labeling index for tumor epithelia. Mean ± SD ($n = 7$) is shown. (*F*) Alcian blue staining. Tumors with shAPCs+shPTEN were stained. Mucus pools stained in blue (closed arrowhead) in stroma (*Upper*). *Insets* are enlarged in lower panel. Mucus and cellular debris shed into the lumen (*Lower Left*) are leaking (open arrowhead) from the disrupted glands (*Lower Right*). (Scale bar, 50 μm.)

shAPCs alone comprised nodules resembling Matrigel plugs, but having focal white spots inside (Fig. 3*A*). We classified them as "nontumor," based on too low a proportion of epithelial cells. Thus, tumors were tentatively defined as nodules replacing coinjected Matrigel with proliferating epithelial glands at 6 wk after injection. By applying this criteria, the tumor development rate by shAPCs alone was 63% (=7/11), 5 cases for both sides and 2 cases for either side, among 11 cases (Table 1). These results suggested that *APC* suppression might be integral but not always sufficient for tumor development from organoids, consistent with earlier studies in vivo (17, 18, 32).

Suppression of *p53* or *PTEN* Promotes *APC*-Dependent Tumorigenesis from Organoids. Many gene-modified mice have been crossed with *APC* mutant mice to evaluate their impact on carcinogenesis, in which common readouts were multiplicity, size, and histology of the tumors. We wondered if similar analysis could be feasible at the cellular level. By cointroducing shp53 or shPTEN with shAPCs into organoids (Fig. 2*A*), tumor development was observed for both sides of nude mice in all of the cases tested (Fig. 3*A*; Table 1). Similar results were obtained by introduction of shAPCs into *p53*- (Fig. S3) and *PTEN*-deficient organoids (Table 1). A significant increase in tumor size was also observed (Fig. 3*D*), but an increase was not observed in proliferation index (Fig. 3*E*). No remarkable effects were detected in histological features, including mucus pool formation (Fig. 3*F*), tumor gland morphology (Fig. S4*A*), and β-catenin accumulation (Fig. S4*B*). Taken together, cooperation for tumorigenesis between *APC* loss and inactivation of either *p53* (9, 10) or *PTEN* (11, 12) could be recapitulated in tumors as an increase in size and development rate. We also verified that organoid culture was conducted in a stromal cell–free condition (Fig. S5), confirming tumorigenesis was indeed achieved with IECs alone.

Significant Acceleration of *APC*-Dependent Tumorigenesis from Organoids by *Kras* Activation. To reconstitute somatic mutation of *Kras*, which is frequent in human CRC (19), we deleted a stop codon flanked by two loxP elements [Lox-Stop-Lox (LSL)] blocking the expression of $Kras^{G12D}$ by lentiviral Cre-mediated recombination (33) in IECs from $Kras^{LSLG12D/+}$ mice (34, 35). Successful deletion was confirmed by detecting the "1-loxP" fragment (35) in genomic PCR (Fig. 4*A*). Amplification of the LSL cassette revealed its partial and complete deletion in organoids with Cre and shAPCs+Cre, respectively (Fig. 4*A*). On $Kras^{G12D}$ expression, active Ras enriched (Fig. 4*B*) without affecting the morphology of the organoids (Fig. 4*C*), verifying specific activation of Ras but not the Wnt pathway. We then asked whether a synergy between oncogenic *Kras* and *APC* loss in intestinal tumorigenesis (13–15) could be recapitulated in our model. Strikingly, organoids with shAPCs+Cre gave rise to tumors on both sides so rapidly that the nude mice became moribund at 2 wk after injection (Fig. 4*D*) in 11 of 11 cases (Fig. 4*E*). They typically appeared red, indicative of active angiogenesis and hemorrhage, and contained cystic dilatation due to retention of serous fluid (Fig. 4*D*). Compared with organoids with shAPCs alone, a significant increase in tumor size was observed (Fig. 4*F*). Cre did not synergize with shAPCs in $Kras^{+/+}$ organoids (Fig. S6), ruling out the possibility of direct synergy between Cre and shAPCs. Tumor glands became more densely packed with morphological alteration from an irregular cystic structure (Fig. 5 *B* and *E*) to a tubular or papillary structure (Fig. 5 *C* and *F*). Destruction of glands leading to mucus pool formation (Fig. 5*H*) disappeared, despite retained mucus production ability (Fig. 5*I*). Given no effects on both cell proliferation (Figs. 4*G* and 5 *K* and *L*) and the magnitude of β-catenin accumulation (Fig. 5 *N* and *O*), $Kras^{G12D}$ might have induced tumor growth through histological alterations. Thus, the synergy was successfully recapitulated in tumors as an increase in size and development rate and alteration in histology.

Marginal Effects by *Kras* Activation Alone on Tumorigenesis from Organoids. We also characterized nodules with either of shAPCs or $Kras^{G12D}$ at 2 wk postinjection for reference, although this was too early for the correct diagnosis. If the criteria for tumors were automatically applied, tumor-positive cases were seven of seven for shAPCs, three of seven for $Kras^{G12D}$, and zero of seven for pLKO.1 (Fig. 4*E*). Putative tumors induced by $Kras^{G12D}$ contained

Table 1. Summary of tumor development induced by shRNA transduction

Genes/genotypes		V	P	5	5P	A	A5	AP	A5P
shRNA	shAPCs					+	+	+	+
	shp53			+	+		+		+
	shPTEN		+		+			+	+
	pLKO.1	+							
IEC	WT	0/14	0/4	0/5	0/3	7/11	8/8	10/10	1/1
	p53 $^{-/-}$	0/4	0/2	—	—	2/2	—	4/4	—
	PTEN $^{-/-}$	0/1	—	—	—	—	1/1	—	—

—, not tested. V, P, 5, and A depict vector, shPTEN, shp53, and shAPCs, respectively.

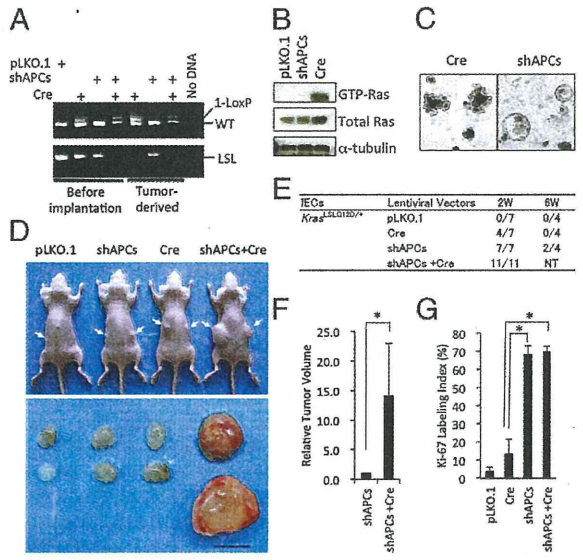

Fig. 4. Synergy between *APC* suppression and *Kras* activation in organoids for tumorigenesis. (*A*) Cre-mediated recombination in vitro. Genomic PCR analysis for WT and recombined allele of *Kras* (*Upper*) and for the LSL cassette (*Lower*). 1-LoxP, single LoxP after the recombination. (*B*) Enrichment of active Ras by induction of $Kras^{G12D}$. Immunoblotting analysis for Ras before (*Middle*) and after (*Top*) GST pulldown assay. α-Tubulin serves as a loading control (*Bottom*). (*C*) Transduced organoids in 3D culture. $Kras^{G12D}$ did not induce cystic shape. (*D*) s.c. tumors in nude mice. Palpable nodules (arrow) at 2 wk postinjection (*Upper*) and corresponding nodules after excision (*Lower*). (Scale bar, 10 mm.) (*E*) Summary of tumor development. Data at 2 and 6 wk after the implantation are shown. NT, not tested. (*F*) relative ratio of tumor volume. Mean ± SD (n = 7 each) is shown, $*P < 0.01$. (*G*) Ki-67 labeling index for epithelia in the nodules. Mean ± SD is shown; $*P < 0.01$. pLKO.1 (n = 3), Cre (n = 3), shAPCs (n = 7), and shAPCs+Cre (n = 7).

a few glands of ductal or cystic shape (Fig. 5 *A* and *D*), with impaired cell differentiation and proliferation as exemplified by loss of mucus production (Fig. 5*G*) and low Ki-67 index (Figs. 4*G* and 5*J*), respectively. Consistent with the lack of Wnt pathway activation (Fig. 4*C*), β-catenin remained in the membrane (Fig. 5*M*). $Kras^{G12D}$ nodule-derived organoids proved completely deleted for LSL, which had been only partially deleted when injected (Fig. 4*A*), suggesting their transient growth advantage. However, $Kras^{G12D}$ tumors no longer remained at 6 wk after injection (Fig. 4*E*), indicating that *Kras* activation by itself was insufficient for establishment of tumors, consistent with previous studies reporting no effect in the small intestine (13, 14, 36) and induction of only hyperplasia in the colon (15, 37). Also, initial proliferation and eventual extinction might mirror the natural course of aberrant crypt foci (ACF) (38), which are early lesions of the colon highly associated with *Kras* mutation (39).

Acquired Cancer Stem Cell–Like Properties in Tumor-Derived Organoids. As intestinal stem cells (ISCs) were unable to survive in s.c. tissue (Fig. 3*A*), development and maintenance of tumor glands with differentiated and proliferative properties (Fig. 3 *C* and *F*) suggested the emergence of a distinct subpopulation with the ability to self-renew and differentiate. To better characterize the nature of induced tumors, we harvested all of the nodules to conduct organoid cultures and obtained organonids only from tumors. *APC* and *PTEN* were suppressed by corresponding shRNAs (Fig. 6*A*), confirming successful transduction. Tumor-derived organoids proved tumorigenic in all seven cases examined. Notably, tumors from identical cells gave rise to secondary tumors akin to the primary tumors in both magnitude (Fig. 6*B*) and histology (Fig. S7*A*), regardless of genetic background (Fig. S7*B*), further implying the emergence of a cancer stem cell (CSC)-like subpopulation. Sphere-forming potential in suspension culture has been associated with stemness (40). Whereas single cells containing ISCs did not form spheres, tumor-derived organoids yielded spheroids (Fig. 6*C*) in all seven cases examined. Even never-implanted organoids with shAPCs alone formed spheroids (Fig. 6*C*), suggesting induction of the CSC-like properties even before injection into nude mice. Quantitative PCR (qPCR) analysis revealed up-regulation of CSC markers *CD44* and *CD133* (41) but not ISC markers *Lgr5* or *Bmi1* (42) in tumor-derived organoids (Fig. 6*D*). Despite up-regulation of *Axin2* and *CD44* in tumors, *c-Myc* or *CCND1* were not induced, suggesting selective activation of a subset of Wnt target genes toward acquisition of CSC properties. Taken together, these results supported the notion that ISC-containing organoids likely comprised a subpopulation with CSC-like properties through *APC* inactivation.

Discussion

To model human CRC, inactivation of *APC* and subsequent tumor development in the intestine have basically been achieved in mutant or gene-modified mice for *APC* (4). In contrast, we demonstrated that it could also be achieved without a genetically

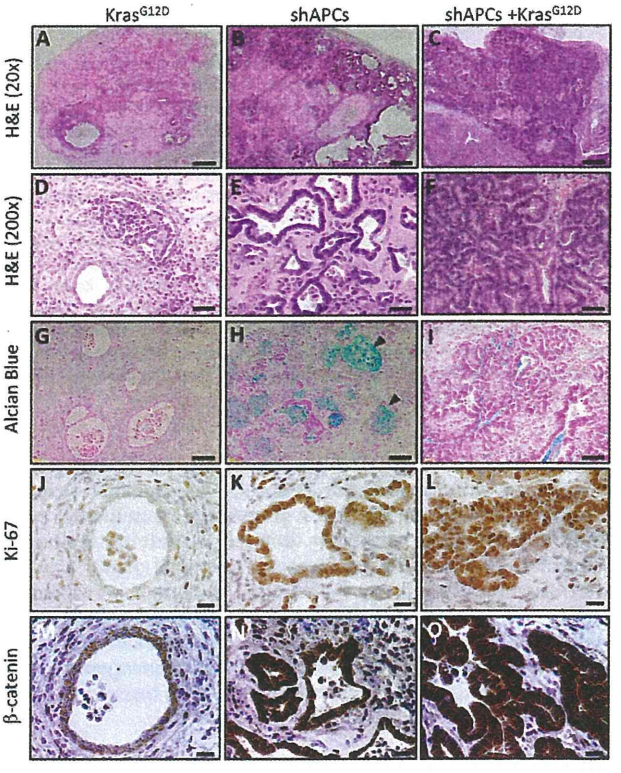

Fig. 5. Histological features of the tumors induced from $Kras^{LSL\text{-}G12D/+}$ organoids. (*A–F*) H&E staining at 20× (*A–C*) and 200× (*D–F*) magnification. (Scale bar, 500 and 50 μm, respectively.) (*G–I*) Alcian blue staining. No mucus production (*G*), formation of multiple mucus pool (closed arrowheads) in the stroma (*H*), and mucus confined in the lumen of intact glands (*I*). (Scale bar, 100 μm.) (*J–L*) Immunostaining for Ki-67. Few (*J*) and many (*K* and *L*) positive cells in the tumor glands are observed. (Scale bar, 25 μm.) (*M–O*) Immunostaining for β-catenin. Localized in the membrane (*M*), and accumulated in the cytoplasm or nucleus (*N* and *O*). (Scale bar, 25 μm.) Representative images are shown. Tumors were generated by $Kras^{G12D}$ (*Left*), shAPCs (*Center*), or both (*Right*) from identical cells and harvested at 2 wk after injection.