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.

\textsc{animals} | \textsc{carcinogenesis} | \textsc{primary culture} | \textsc{Matrigel} | \textsc{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 \textit{CTNNB1} gene encoding \(\beta\)-catenin result in \(\beta\)-catenin accumulation through inhibition of its degradation, leading to constitutive activation of the Wnt pathway that is transcriptionally regulated by the \(\beta\)-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 predominately 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 \textit{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 (\textit{PTEN}) (11, 12) or induction of oncogenic \textit{Kras} (13–15) significantly promoted intestinal tumorigenesis only in the context of \textit{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 underling tumorigenesis were also demonstrated by genetic ablation of \textit{Mvyd88} (17) and \textit{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 genes, we added Matrigel to the lentiviral transduction media, but the infection efficiency was still unsatisfactory. To solve this problem, we added Matrigel directly into the lentiviral transduction media, which resulted in the efficient transduction of intestinal epithelial cells (IECs). The results are shown in Fig. 1.

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However, we assumed that this might not be necessarily the case, documented for organoids from APC (27), suggesting a link between the morphology and time (Fig. 2D), which was also confirmed by qPCR analysis demonstrating up-regulation of Axin2 (2,2E), 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. 3A). They were characterized by epithelial glands and prominently infiltrated stromal cells (Fig. 3B). Active proliferation of epithelia was verified by high Ki-67 labeling index and inferred from β-catenin accumulation (Fig. 3C). 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. 3A). As they histologically lacked epithelial glands (Fig. 3B), 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. 3S; Table 1), in line with earlier studies in vivo (11, 12, 30, 31). In some cases, organoids with shAPCs in more detail. We found that they were puro-resistant and indeed suppressed for expression of APC (Fig. S2D). In thin sections, they lost physiological properties such as polarity (Fig. 2B), differentiation (Fig. 2C), and cellular turnover (Fig. 2B 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. 2D), which is shown. Mean ± SD (n = 3) is shown.

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. S2A) individually into organoids. With a routine schedule for 3D culture (Fig. S2B), however, we frequently failed in propagation for any shAPC clone tested, even under drug selection (Fig. S2C), suggesting adverse effects by APC knockdown in vitro. In contrast, introduction of potent shp53 or shPTEN (Fig. S2D) resulted in steady propagation of organoids (Fig. S2A). Which spontaneously became puro-resistant, suggesting a growth advantage of inactivating p53 or PTEN. We later found that co-introducing 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. 2A; Fig. S2C). 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.

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.

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.
was not observed in proliferation index (Fig. 3).

βTumors with shAPCs alone stained for

features of the nodules. H&E staining of Matrigel plugs (Fig. 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. 3A). We classified them as “nontumor,” based on too low a proportion of epithelial cells. Thus, tumors were tentatively defined as nodules replacing coinfected 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 coinroducing shp53 or shPTEN with shAPCs into organoids (Fig. 2A), tumor development was observed for both sides of nude mice in all of the cases tested (Fig. 3A; 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. 3D), but an increase was not observed in proliferation index (Fig. 3E).

Table 1. Summary of tumor development induced by shRNA transduction

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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 KrasG12D by lentiviral Cre-mediated recombination (33) in IECs from KrasLSLG12D/+ mice (34, 35). Successful deletion was confirmed by detecting the “1-loxP” fragment (35) in genomic PCR (Fig. 4A). Amplification of the LSL cassette revealed its partial and complete deletion in organoids with Cre and shAPCs+Cre, respectively (Fig. 4A). On KrasG12D expression, active Ras enriched (Fig. 4B) without affecting the morphology of the organoids (Fig. 4C), 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 by 2 wk after injection (Fig. 4D) in 11 of 11 cases (Fig. 4E). They typically appeared red, indicative of active angiogenesis and hemorrhage, and contained cystic dilatation due to retention of serous fluid (Fig. 4D). Compared with organoids with shAPCs alone, a significant increase in tumor size was observed (Fig. 4F). 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. 5B and E) to a tubular or papillary structure (Fig. 5C and F). Destruction of glands leading to mucus pool formation (Fig. 5H) disappeared, despite retained mucus production ability (Fig. 5I). Given no effects on both cell proliferation (Figs. 4G and 5K and L) and the magnitude of β-catenin accumulation (Fig. 5N and O), KrasG12D 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 KrasG12D 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 KrasG12D, and zero of seven for pLKO.1 (Fig. 4E). Putative tumors induced by KrasG12D contained

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— not tested. V, P, S, and A depict vector, shPTEN, shp53, and shAPCs, respectively.
of induced tumors, we harvested all of the nodules to conduct analysis for WT and recombined allele of \(Kras\) \(^{G12D}\), which had been only partially deleted when injected (Fig. 4).

Acquired Cancer Stem Cell-like Properties in Tumor-Derived Organoids. As intestinal stem cells (ISCs) were unable to survive in s.c. tissue (Fig. 3A), development and maintenance of tumor glands with differentiated and proliferative properties (Fig. 3C 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 organoids only from tumors. \(APC\) and \(PTEN\) were suppressed by corresponding shRNAs (Fig. 6A), 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. 6B) and histology (Fig. S7A), regardless of genetic background (Fig. S7B), 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 spheres (Fig. 6C) in all seven cases examined. Even never-implanted organoids with shAPCs alone formed spheres (Fig. 6C), 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. 6D). 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
IECs transduced with shAPC frequently failed to propagate, which might be partially in line with adverse effects caused by acute APC loss (28). We incidentally noted that this could be overcome by co-introducing all of the shAPC clones. A possible explanation could be that pooled shAPC clones yielded variations in the magnitude of Wnt activation, thereby increasing the probability of achieving the “just-right” signaling (46). Alternatively, cooperation among off-target effects by pooled clones could have contributed. Although the underlying mechanism remains to be investigated, organoids that grew out indeed carried shAPCs and phenocopied those derived from APC-deficient adenoma. Based on the high similarity, we reasoned that propagated organoids with shAPCs might likely be an in vitro equivalent to adenoma. We then took advantage of this situation for further analysis.

The third was the strict definition of tumor in this model, which was definitely required to relate the results to earlier studies in vivo. We tentatively defined tumors as nodules replacing co-injected Matrigel with proliferating epithelial glands at 6 wk post-injection. Only if tumors proved lethal at an earlier point were nodules exceptionally diagnosed on death. Accordingly, nonlethal nodules at 2 wk after injection were not treated as tumors. Acquisition of the potential for sphere formation and serial transplantation and induction of CSC markers were confirmed in tumor-derived organoids, clearly indicating that they had indeed undergone transformation. These results tend to support the validity of our definition of tumors.

With this experimental system, the relevance of known genetic alterations in CRC could be essentially recapitulated, either individually or in the context of APC loss, as in PTEN loss (11, 12) and Kras activation (13–15, 36, 37). With regard to p53 loss, its genetic cooperation with APC loss was negative in the heterogeneous genetic background (31, 47) but proved to be positive in a congenic background (9, 10), consistent with this study. These findings highlight the relevance of conducting the analysis on genetic interaction in exactly the same genetic background as achieved in our model, which otherwise requires multiple back-crossing. Thus, validation of candidate genes or genetic cooperation will be warranted, leading to quick identification of the genes to be prioritized for further investigations from many candidates (19, 20) before, or even without, generation of genetically clean mouse lines and cell lines. Candidate genes or genetic cooperation of tumors with defined and colon cancer might then be facilitated. The custom-made “genetically clean” cell lines would become valuable resources for identification of effective compounds or therapeutic targets by high-throughput screening or in preclinical studies.

Considering similarities in outcome and approach, our in vitro model might well be comparable to those two types of in vivo studies, in which APC was subject to acute deletion in the intestine, rather than studies with the APC-heterozygous mutant mouse (15). One is APC loss in an Lgr5+ stem cell–specific manner, which quickly gives rise to adenoma (22, 26, 48). The other is APC loss either focally or entirely in the intestine. Local injection of adenovirus-Cre induced adenoma in the distal colon, although transduction was achieved in only a limited area (44, 45). Cre-mediated inducible and acute loss of APC throughout the intestine led to morbidity within 5 d (28), but the crypts that were rescued by harvesting 2 d after APC loss proved tumorigenic in nude mice (49). In both cases, APC inactivation was achieved in gene-modified mice in vivo, presumably by cooperating with the microenvironment. Besides, special conditions such as cell type–specific gene ablation or crypt harvest at specific times were necessary. In contrast, in our model, APC inactivation was simply achieved in WT IECs in vitro, without any other type of cells or experimental conditions, obviously facilitating intestinal tumorigenesis studies. As organoid culture is optimized for Lgr5 stem cells (24), it is conceivable that Lgr5+ stem cells were predominantly transformed in our model. On the other hand,
lentiviral transduction could also target quiescent stem cells, which could be marked by Bmi1 (42), Lgr1 (50), mTert (51), or HopX (52), in a mutually overlapping but distinct manner. Moreover, even Lgr5\(^+\) differentiated cells could de-differentiate to reacquire stem cell properties and initiate tumorigenesis (49). Thus, there is a possibility that tumor initiation could take place through many different pathways, including reprogramming of nonstem cells. In this regard, our model might provide unique opportunities in addressing this issue in an unbiased way, as the entire process of tumorigenesis could be simply recapitulated without predefined conditions on tumor-initiating cells.

In conclusion, we developed a unique in vitro model for CRC, with which genetic interactions in both tumor initiation and progression will be simply but genuinely analyzed. By serving as an alternative or complement to the standard approaches, it would likely accelerate CRC research.

Materials and Methods

Singly dissociated intestinal cells were lentivirally transduced in vitro. Organoids were maintained for 4 wk in Matrigel and injected into nude mice to evaluate tumorigenicity. Several weeks after the implantation, the tumors were embedded to histological analysis or 3D culture to obtain a pure population of tumor-derived organoids, which were further analyzed by Western blotting, qPCR, and sphere-forming assay. Extended materials and methods are available in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Mice and Animal Studies. C57BL/6j mice and immune-deficient nude mice, BALB/cαnu/nu were purchased from CLEA Japan. Mice homozygous for the p53mut allele were obtained by crossing mice heterozygous for the mutant p53, and genotyping was performed as previously described (1). Conditional knock-in mice heterozygous for the Lox-STOP-Lox-KrasG12D allele or LSL-KrasG12D mice (Jackson Laboratory) were maintained by back-crossing with C57BL/6j mice. Genotyping and confirmation of Cre-mediated removal of the Stop codon in the Lox-Stop-Lox (LSL) cassette were performed as previously described (2). Mice carrying floxed alleles of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) (3) were intercrossed with VhlCre transgenic mice (Jackson Laboratory) to generate intestinespecific conditional KO mice. Animal studies were carried out according to the Guideline for Animal Experiments, drawn up by the Committee for Ethics in Animal Experimentation of the National Cancer Center, which meet the ethical standards required by the law and the guidelines about experimental animals in Japan.

Isolation of Intestinal Crypts and 3D Organoid Culture. The small intestine was separated from mice at 3–5 wk of age. After several rounds of washes with cold PBS, tissues were chopped into 5-mm pieces. Intestinal crypts were selectively isolated essentially as previously described (4), but some modifications were made to the subsequent procedures and media in organoid culture. Briefly, crypts were further dissociated into single cells by Accutase (Innovative Cell Technologies) treatment for 10 min at 37 °C and seeded on polymerized Matrigel (BD Biosciences). Accordingly, the serum-free media optimized for organoid culture containing EGF (Peprotech), R-Spondin1 (R&D), and Noggin (Peprotech) (4) were routinely supplemented with Y27632 (Wako) and Jagged-1 (AnaSpec) to support the survival of single cells and proliferation of stem cells (4). These modified culture media were used for all of the experiments throughout this study, including viral infection and the organoid culture of normal, transduced, and tumor-derived cells, to provide identical culture conditions, although transformed cells in fact required fewer factors for survival (5). The dissociated single cells were re-suspended in the culture media, seeded on 80 μL Matrigel/well in a 12-well plate, and incubated overnight at 37 °C. The next morning, floating dead cells and the media were removed. Viable cells attached onto Matrigel were covered with 70 μL Matrigel and overlaid with the media to resume 3D culture. Typically, one 12-well plate was appropriate to start the primary culture of single cells from the intestines of one to two mice. Subculture was conducted every 7–10 d at 1:1–1:3. In each subculture, organoids were directly harvested with a cell scraper, washed with PBS, and dissociated into single cells by Accutase treatment and vigorous pipetting to eliminate debris or dead cells accumulated inside the organoids. Single cells were seeded on Matrigel for overnight incubation, and only viable attached cells, typically ~10% of the total harvested cells, were subject to subsequent 3D culture, as described above. Viability was assessed with Trypan blue staining for single cells, before seeding or after attachment to Matrigel. For spheroid formation assay, singly dissociated 10^5 cells/mL were plated in a 24-well multidish with low cell binding (Thermo Fisher Scientific) and maintained in organoid culture media for 3 wk with periodic medium change.

Lentiviral Vectors and Infection. For shRNA transduction, pLKO.1-puro vectors (Sigma-Aldrich) targeting murine PTEN (TRCN28992), p53 (TRCN12359), and adenomatus polyposis coli (APC) (TRCN42533-7) were used. Each shRNA against APC (shAPC) clone TRCN42533-7 corresponds to clone 1–5 of shAPC in Fig. S2. To make a pool of the shAPC clones, a one-fifth amount of each clone was equally mixed for transfection to produce viral particles. LV-Cre pLKO.1 (Addgene plasmid 25997), encoding Cre-recombinase in the backbone of pLKO.1 vector (6), was used for in vitro removal of the Stop codon flanked by two LoxP sequences. pCDH-CMV-MCS-E1-CopGFP (System Biosciences) and pLKO.1-puro were used as a GFP-expressing vector and non-GFP-expressing vector, respectively. The GFP-expressing vector was always included along in the infection experiments to monitor transduction efficiency, which was evaluated 48 h after the infection by counting the GFP-positive organoids under the microscope. Because this vector does not carry any drug-resistant genes, selection before the counting was not conducted. Lentiviral particles were generated using the ViraPower Lentiviral Expression System (Invitrogen) following the manufacturer’s instructions. The collected viral supernatants were concentrated 10-fold with PEG-it Virus Precipitation Solution (System Biosciences), passed through a 0.45-μm filter, and stored at −80 °C until used for infection. For lentiviral infection, 1–5 × 10^5 single intestinal cells, dissociated immediately after isolation from mice or after 1-wk primary 3D culture, were resuspended in 500 μL of 1.5X culture media supplemented with 3.75 μL of TransducX (System Biosciences) and mixed with 250 μL of 10-fold concentrated viral particles. They were seeded on polymerized Matrigel in a 12-well plate and incubated overnight at 37 °C, followed by the same procedures for subculture. For double infection, 125 μL each of viral particles containing each lentiviral construct was mixed to make up 250 μL. Puromycin selection (2 μg/mL) in 3D culture for the pLKO.1-based vectors was conducted for 3–4 d only when complete transduction needed to be assured, such as in evaluating knockdown efficiency of each shRNA in intestinal organoids by Western blotting, or qPCR analysis of Axin2 and thin section studies in organoids with shAPCs, but not in the experiments related to tumor development.

Tumorigenicity Assay in Nude Mice. After the lentiviral transduction, intestinal cells were propagated in 3D culture for 4 wk. We basically maintained transduced organoids without drug selection, because knockdown of tumor suppressor genes per se proved to be positively selected in 4 wk. This advantageous situation also allowed us to avoid puromycin-induced possible morphological transition, by which we could always assure that induction of cystic organoids was specifically achieved by shAPCs. Organoids grown in Matrigel were directly harvested with a cell scraper, repeatedly washed, and resuspended in PBS. An aliquot of 1/10th volume was completely dissociated with Accutase for cell counting with Trypan blue. Organoids corresponding to 5 × 10^6 cells were mixed with 200 μL Matrigel and injected into one side of the dorsal skin of nude mice. After 2–6 wk, palpable tumors or residual Matrigel plugs, if any, from the injected sites were harvested for histological examination or cell culture. The volume (V) of each tumor was calculated by the formula: V = 1/2 × (width)^2 × (length). For comparison of tumor volume, only the cases with tumor development in both sides of nude mice were included in the analysis, in which the mean value for volume of the tumors in both sides was used. The tumors generated by co-introduction of shAPCs and other shRNA or Cre were compared with the tumors simultaneously generated by shAPCs alone in the same experiment to calculate the relative ratio of tumor volume. The results were statistically analyzed by
paired Student t test. To recover tumor-derived epithelial cells, s.c. nodules were minced into 2-mm pieces and digested with 2 U/mL dispase II and 1 mg/mL collagenase P (Roche Diagnostics) for 30 min at 37 °C. Single cells were obtained by passing through a 40-μm cell strainer (BD Biosciences) and seeded on polymerized Matrigel. Only attached cells were subject to subsequent 3D culture as described above. Because the serum-free culture media do not support survival of tumor-derived stromal cells, a pure population of epithelial cells was normally obtained within 10 d.

Western Blotting. Intestinal organoids in 3D culture were harvested after lysis of Matrigel with Cell Recover Solution (BD Biosciences) for 1 h on ice. The organoids were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA). Protein concentrations were quantified with a Pierce BCA protein assay kit (Thermo Fisher Scientific), and 5–10 μg was resolved by SDS/PAGE under reducing conditions in regular Tris-glycine buffer on a gradient gel SuperSep Ace 5–20% (Wako). The proteins were electrically transferred in a wet-tank to a PVDF membrane. After blocking with 4% Block Ace (DS Pharma Biomedical), the membranes were incubated overnight at 4 °C with primary antibodies. The antibodies used in this study were c-Myc (Abnova) for APC, 1C12 for p53, 138G6 for PTEN (Cell Bioscience), the membranes were incubated with a corresponding secondary antibodies for 1 h at room temperature and finally visualized by Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific). Knockdown efficiency was evaluated 1 wk after lentiviral transduction without drug selection. To induce p53 protein, organoids were treated with 2 μg/mL of adriamycin 24 h before harvest. For detection of GTP-Ras, an activated form of Ras, a Raf-GST pull-down assay was performed with 140 μg of total cell lysates using a Ras activation kit (Enzo Life Sciences).

RNA Extraction and RT-PCR. Total RNAs were extracted from the organoids with TRIzol (Invitrogen), and 500 ng of total RNA was reverse transcribed with high-capacity cDNA Reverse Transcription Kits (Applied Biosystems). In quantitative RT-PCR (qRT-PCR) analysis, each cDNA was subsequently amplified with Power Syber Green PCR Master Mix in the 7300 Realtime PCR System (Applied Biosystems), following the manufacturer’s instructions. Forward and reverse primers used for specific amplification of each gene’s transcript were as follows—Axin2: aagagaagacacaccaatac and etgatgecatctetctetg; Lgr5: tggaaacaaaggtgga and ggcacctgatgtggt; Cend1:agaccttgggctcctgt and cagttcaggtcttcct; e-Myc: agtggttctgagagacac and ggtttgctctctccacag; CD133: tgcctctctctaaatgg and tggattcaagttctgtct; CD44: gtagttcctcctgtct and ctggttaggcctattttctc. Knockdown efficiency was statistically analyzed by Student t test. In standard RT-PCR analysis, cDNA was amplified with AmpliTaq Gold (Applied Biosystems). GAPDH was amplified as previously described (7). Other primers used are as follows—EpCAM: gagagactgtctgtgtacacaa and ggtggtagacacacca; Vimentin: gagctgggagcgattc and ccacctcagaggtgact; α–SMA: gtatcagctgaaagca and cccctgagaggtgtga.

Histopathology. All of the s.c. tumors were formalin fixed, paraffin embedded, and sectioned at 5 μm. Organoids in Matrigel were selected under the microscope and fixed overnight with paraformaldehyde. Fixed organoids were collected and embedded first in agarose gel and subsequently in paraffin. H&E staining was used for standard histology analysis. To characterize mucus in the tumor tissues, Alcian blue staining was conducted, with nuclear counterstaining by Nuclear Fast red. For immunohistochemical analysis, after antigen retrieval by autoclave, anti–β-catenin rabbit mAb 6B3 (Cell Signaling Technology) and anti–Ki-76 rabbit polyclonal antibody (Novocastra) were used as the primary antibodies at a dilution of 1:500 and 1:1,000, respectively. Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as the secondary antibody at a dilution of 1:200. Specific signals were visualized with the Vectastain Elite ABC system (Vector Laboratories). Ki-76 labeling index was calculated by counting the number of cells with positive nuclear staining among more than 1,000 cells per slide. The results were statistically analyzed by Student t test.

Fig. S1. Viability of intestinal epithelial cells (IECs) after dissociation of organoids and attachment to Matrigel. Viability of total harvested cells (Left). Viable cells were counted immediately after dissociation of organoids under 3D culture for 1 wk after isolation from the intestine. Viability of Matrigel-attaching cells (Right). Viable cells were counted at 16 h postseeding. The proportion to seeded $5 \times 10^4$ viable cells is shown. The presence of viral particles did not affect viability of IECs. Mean ± SD ($n = 3$) is shown. NS, not significant.

Fig. S2. RNAi-mediated knockdown of tumor suppressor genes. (A) Knockdown efficiency by shRNA against APC in mouse embryonic fibroblast (MEF). A total of five shRNA clones against APC were tested, individually or as a pool. The protein level of APC was analyzed by immunoblotting. pLKO.1 is an empty vector for shRNA constructs. α-Tubulin serves as a loading control. (B) Time schedule of cell culture after lentiviral transduction. (C) Immunoblotting analysis for APC, PTEN, and p53 in organoids. Robust knockdown of tumor suppressor genes (TSGs) by corresponding shRNAs was confirmed. To verify knockdown of p53, transduced organoids were treated with adriamycin (2 μg/mL) 1 d before the analysis to activate p53 protein. α-Tubulin serves as a loading control. (D) Organoids transduced with shAPC(s) in 3D culture. All of the cells were subcultured simultaneously and plated at the same dilution ratio. Representative results at days 6, 14, and 23 are shown. Large rounded cysts emerged and dominated the cell population for organoids transduced with the pool (shAPCs) but not with individual clones of shAPC.
**Fig. S3.** Induction of tumors from p53-deficient organoids. H&E staining at 20x (Left) and 200x (Right) magnification. (Scale bar, 500 and 50 μm, respectively.) The nodules from p53-deficient organoids with pLKO.1 do not contain viable epithelial glands (A and B). Tumors induced by shAPCs (C and D) and shAPCs+shPTEN (E and F) contain tubular epithelial glands accompanied by massive stromal infiltration. Representative images are shown. (Scale bar, 100 μm.)

**Fig. S4.** Induction of tumors from WT organoids. Histological features of tumors induced from intestinal organoids transduced with shAPCs+shp53 (Upper) and shAPCs+shPTEN (Lower) are shown. No significant changes in histology were observed by cointraduction of shp53 or shPTEN with shAPCs. Differentiated features such as mucus secretion and cellular turnover and partial loss of β-catenin accumulation were similarly observed. (A) H&E staining at 20x (Left) and 200x (Right) magnification. (Scale bar, 500 and 50 μm, respectively.) (B) Immunohistochemical analyses of Ki-67 (Left) and β-catenin (Right). Representative images are shown. (Scale bar, 25 μm.)
Table 1 shows the expression levels of various markers in different samples before and after organoid culture. The expression of EpCAM, Vimentin, α-SMA, and GAPDH was assessed using RT-PCR analysis. The absence of stromal cells in organoid culture was confirmed by the undetectable expression of Vimentin and α-SMA in the organoids with shAPCs at day 35, the timing when organoids are typically injected into nude mice. GAPDH served as an internal control.
Synergy between APC suppression and Kras activation but not Cre. (A) Nude mice at 3 wk after injection of the transduced organoids. (B) Excised s.c. tumors. Development of huge tumors was induced by transduction with Cre and shAPCs from Kras^{LSL-G12D/+} but not from Kras^{+/+} organoids. The tumors were dilated due to serous fluids or internal hemorrhage (A). Flattened tumors due to rupture during the excision procedure (B). (Scale bar, 10 mm.)
Fig. S7. Secondary tumors from tumor-derived organoids. (A) H&E staining at 20× magnification of T19 (shAPCs) and T21 (shAPCs+shPTEN). Primary and secondary tumors were histologically indistinguishable. (Scale bar, 500 μm.) (B) Secondary tumors by reimplantation of tumor-derived organoids. T7 (shAPCs)/T4 (shAPCs+shPTEN) and T8 (shAPCs)/T9 (shAPCs+Cre), derived from the same p53-deficient IECs (left) and Kras^{G12D/+} IECs (right), respectively, were retransplantable in nude mice. Enlargement of the tumors by introduction of shPTEN or activation of Kras^{G12D} is reproduced in secondary tumors as well. (Scale bar, 10 mm.)