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 $\times$ 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 $\times$ 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 $\pm$ SD ($n = 3$) is shown.

particles, we coincubated dissociated single cells and viral particles on Matrigel (Fig. 1A), which achieved high transduction efficiency (Fig. 1B). About half of the cells composing organoids were viable, and $\sim 20\%$ 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. 1B), 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. 1C), 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. 1B). 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. S24) 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 knock-down in vitro. In contrast, introduction of potent shp53 or shPTEN (Fig. S2D) resulted in steady propagation of organoids (Fig. 2A), which spontaneously became puromycin-resistant, suggesting a growth advantage of inactivating p53 or PTEN. We later found that reintroducing 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 in more detail. We found that they were puromycin-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 was also confirmed by qPCR analysis demonstrating up-regulation of *Axin2* (Fig. 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. 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 μ m.) (E) qPCR analysis of *Axin2* in transduced organoids. Relative expression level of mRNA to β -actin is shown. Mean $\pm$ SD ($n = 3$) is shown; * $P < 0.01$.

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 $\pm$ SD ($n = 7$ each) is shown, $*P < 0.01$. (G) Ki-67 labeling index for epithelia in the nodules. Mean $\pm$ 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. 5A and D), with impaired cell differentiation and proliferation as exemplified by loss of mucus production (Fig. 5G) and low Ki-67 index (Figs. 4G and 5J), respectively. Consistent with the lack of Wnt pathway activation (Fig. 4C), β -catenin remained in the membrane (Fig. 5M). *Kras^{G12D}* nodule-derived organoids proved completely deleted for LSL, which had been only partially deleted when injected (Fig. 4A), suggesting their transient growth advantage. However, *Kras^{G12D}* tumors no longer remained at 6 wk after injection (Fig. 4E), 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. 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 spheroids (Fig. 6C) in all seven cases examined. Even never-implanted organoids with shAPCs alone formed spheroids (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

Fig. 5. Histological features of the tumors induced from *Kras^{SL-G12D/+}* organoids. (A–F) H&E staining at 20 $\times$ (A–C) and 200 $\times$ (D–F) magnification. (Scale bar, 500 and 50 μ m, 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 μ m.) (J–L) Immunostaining for Ki-67. Few (J) and many (K and L) positive cells in the tumor glands are observed. (Scale bar, 25 μ m.) (M–O) Immunostaining for β -catenin. Localized in the membrane (M), and accumulated in the cytoplasm or nucleus (N and O). (Scale bar, 25 μ m.) 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.

Fig. 6. Induction of cancer stem-like properties in tumors through *APC* suppression. (A) *APC* knockdown in tumors. Immunoblotting analysis of tumor-derived organoids for *APC* and *PTEN*. Mouse embryonic fibroblast (MEF) and normal IECs are positive controls. T4/7, T8/9, and T19/21 derived from identical primary cells, respectively. (B) Serial transplantation of tumors. s.c. tumors T19 (shAPCs) and T21 (shAPCs+shPTEN) in primary sites (Left) and corresponding secondary tumors after retransplantation (Right). (Scale bar, 10 mm.) (C) Sphere-forming assay. Representative images after 2-wk suspension culture are shown. (D) qPCR analysis for Wnt target genes and stem cell marker genes. Mean $\pm$ SD ($n = 3$) is shown; * $P < 0.01$; ** $P < 0.05$.

engineered mouse, independently of intestinal microenvironment, and solely with primary IECs, providing an alternative way to model CRC in vitro. There were several issues to be resolved in the course of developing the model. First was to achieve stable transduction of stem cells, instead of gene targeting in embryonic stem (ES) cells. Despite initial technical difficulties, we eventually established a lentivirus-based transduction method, which was as simple as a routine subculture but highly efficient in obtaining stably transduced organoids. This methodological advantage was in sharp contrast to retroviral transduction of only cycling cells, with more complicated procedures but much less efficiency, definitely requiring drug selection to obtain stably transduced organoids (43). Both high infection efficiency and the growth advantage of inactivating tumor suppressors helped us conduct experiments without drug selection, which eliminated its potential side effects and enabled us to use vectors without selection markers or puromycin-resistant IECs from the *Kras*^{LSL-G12D/+} mouse (34). We further showed that Cre-mediated recombination of a floxed allele could be achieved in organoids. Thus, overexpression and knockdown could be simply achieved for many genes in WT IECs and gene disruption in conditionally gene-targeted IECs.

The second was in vitro expansion of *APC*-inactivated organoids. Inactivation of *APC* in the intestine is normally established by a stochastic second hit in heterozygous mice or by complete loss in mice homozygous for a floxed allele in a spatiotemporally regulated manner (22, 44, 45). Although rounded cystic organoids were available by 3D culture of *APC*-deficient adenoma developed in earlier studies (26, 27), *APC* loss and subsequent tumor development have been exclusively achieved in vivo, to which inflammation or interactions within the microenvironment might have played critical roles (17, 18). In some settings, tumor development itself could not be achieved due to organ failure induced immediately after *APC* loss (28). Thus, it was initially unclear whether we could achieve *APC* inactivation in organoids and its subsequent propagation thoroughly in vitro.

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 reintroducing 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 heterozygous 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 backcrossing. 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 gene-modified mice. Also, generation of tumors with defined genotypes could 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), Lgr5 (50), mTert (51), or HopX (52), in a mutually overlapping but distinct manner. Moreover, even Lgr5⁺ differentiated cells could dedifferentiate 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.

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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 subjected 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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