Exploring Tumorigenesis Mechanisms Regulated by The mTOR Pathway Using Human Colon Organoid

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Organoids can bridge the gap between two-dimensional cell lines and in vivo models. They provide an in vitro platform capable of summarizing the complex structure and function of tissues. Organoids, which can mimic the physiological organization and functionality of tissues, have become a valuable tool for disease modeling. In this study, we found that tumor organoids release factors that accelerate the growth of normal organoids and induce them to enter a mesenchymal state. When cultured media containing tumor organoids was treated again with normal organoids, increased proliferation and epithelial to mesenchymal transition (EMT) were confirmed, and the mTOR pathway was also confirmed to be activated at this time. The mTOR (mammalian target of the rapamycin) signaling pathway is a major pathway altered in colorectal cancer (CRC). Abnormal activation of this pathway can affect the progression and metastasis of CRC. Therefore, it is important to find the cause of the abnormally activated mTOR pathway and find a treatment that can target it. Our findings suggest that activation of the mTOR pathway may play a role in increasing proliferation and EMT of normal organoids. It provides evidence for the existence of a tumor microenvironment by suggesting that factors identified in tumor organoid cultured media can contribute to tumorigenesis by activating the mTOR pathway.

Keywords: Organoids, colon cancer, tumorigenesis, mTOR pathway, extracellular vesicles(EV)

Introduction

Colorectal cancer (CRC) is the third most common malignant tumor and the third leading cause of cancer-related deaths. Liver metastasis has been the most frequent and primary cause of death in the patients with CRC, with the overall 5-year survival rate being less than 20%[1]. However, the fundamental mechanisms underlying CRC metastasis to the liver have not yet been fully understood. Therefore, a better understanding of the molecular mechanisms that cause metastasis is urgently and critically needed to improve the survival rates of patients with metastatic disease.

To address such unmet needs, research on human colon organoids is currently ongoing. The development of the intestine organoid system in 2009 was a groundbreaking technological advancement in the field of stem cell research. Unlike previous cell line cultures, this approach utilized background knowledge on the niche components of endogenous intestine stem cells to establish a stable culture system capable of sustaining organoid growth over an extended period.

To form organoids from various tissues, it is necessary to modify the culture methods in a tissue-specific manner, reflecting the lineage commitment factors and niche requirements of the stem cells distributed in each tissue and their differentiated cells. Particularly in the case of CRC organoids that we are dealing with, the addition of various modulators that regulate the WNT (a portmanteau of int and Wg and stands for "Wingless-related integration site") and Notch signaling pathways can proliferate and maintain stem cells[2].

When epidermal growth factor (EGF), R-spondin, and nicotinamide are supplied in Matrigel, cystic organoids are formed. By adding or removing these various niche components, the aim was to establish the most cost-effective culture environment in which organoids can thrive. This approach can lead to a reduction in time and costs associated with organoid culture[3].

The tumor microenvironment (TME) is a complex ecosystem surrounding the tumor, composed of various non-cancerous cells including blood vessels, immune cells, fibroblasts, signaling molecules, and extracellular matrix (ECM). Tumors can influence the microenvironment by releasing extracellular signals, promoting angiogenesis, and inducing peripheral immune tolerance[4]. Conversely, the immune cells within the microenvironment can impact the growth and evolution of cancer cells.

Therefore, we hypothesized that there exists a factor derived from tumor organoids and investigated its impact on the growth of both normal and tumor organoids. The results showed that the addition of tumor organoid cultured media to normal organoids increased growth and enhanced epithelial to mesenchymal transition (EMT). This indicates that the tumor organoid-derived factor X can induce tumorigenesis, particularly by activating

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the mTOR pathway. Consequently, we suggest that this phenomenon may partially explain the tumor microenvironment.

Results

Observation of The Morphology of Tumor Organoid and Normal Organoids

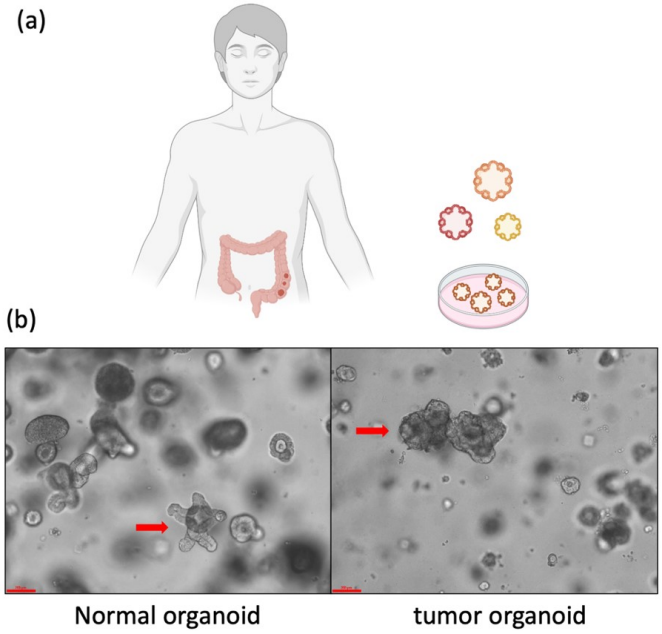

Fig. 1 Examining the morphology of normal and tumor organoids.
(A) schematic illustration of colon organoid.
(B) Brightfield image of normal organoids (*left*) and tumor organoids (*right*) established from the same patient. Left red arrow means the example of a budding structure growing from a human normal colon organoid. Right red arrow illustrate the disorganized morphological appearance of a colorectal cancer.

In our study, we observed the morphology of tumor and normal organoids, utilizing patient-derived colon organoids acquired from the Korea Cell Bank. These organoids were cultured in Matrigel, a medium that supports the maintenance of their 3D structure, facilitating a direct comparison of their morphologies. The normal organoids displayed morphologies that suggested attempts at differentiation, in contrast to the tumor organoids which exhibited more disorganized structures compared to their normal counterparts. Thus, we concluded that normal and tumor organoids possess distinct morphologies. Identifying the microenvironment that drives these morphological differences became our primary objective, highlighting the significance of the tumor microenvironment in cancer research.

Identification of The Characteristics of Organoid

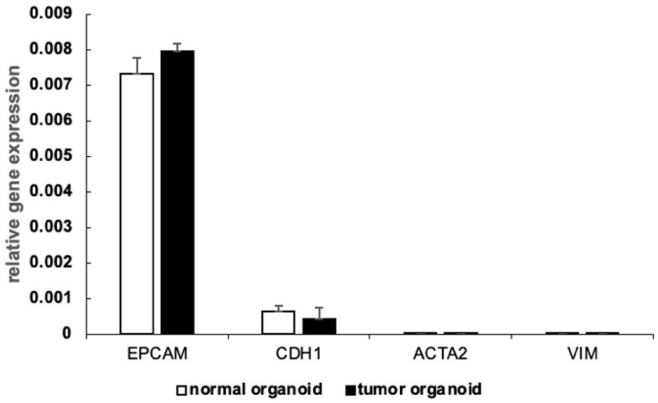

Fig. 2 Gene expression in the normal and tumor organoids.
RT-qPCR analysis of the expression level of EPCAM, CDH1, ACTA2 and VIM in normal and tumor organoid

In this study, we aimed to verify the characteristics of the tumor and normal organoids used by conducting RT-qPCR experiments. Specifically, we assessed the expression of EPCAM and CDH1, markers indicative of epithelial cell type characteristics[5]. As anticipated, both normal and tumor organoids showed high expression levels of these markers, confirming their epithelial nature. Conversely, the expression of fibroblast markers, such as vimentin and ACTA2, was nearly absent, serving as a negative control and indicating the lack of fibroblast characteristics in these samples. Consequently, this demonstrates that both tumor and normal organoids effectively maintain epithelial cell traits in vitro.

Observation of Genetic Background of Tumor Organoid and Normal Organoid

In our study, we aimed to investigate the genetic background of tumor and normal organoids by identifying genetic mutations, thereby understanding the organoid response based on their genetic makeup. It is well-known that common colon tumor organoids harbor mutations in APC, K-ras, and P53 genes[6]. To verify this, we treated the organoids with nutlin-3, a molecule that disrupts the interaction between p53 and MDM2 (mouse double minute 2), and assessed cell viability using a luciferase assay. As expected, normal organoids exhibited strong growth inhibition in response to nutlin-3 treatment, whereas tumor organoids were relatively insensitive to the treatment[7]. This suggests the presence of mutations in p53 and the p53 pathway in tumor organoids. Furthermore, we examined the expression of Axin2, a downstream target of APC, in both tumor and normal organoids. We observed an overexpression of Axin2 in tumor organoids compared to normal ones, indicating mutations

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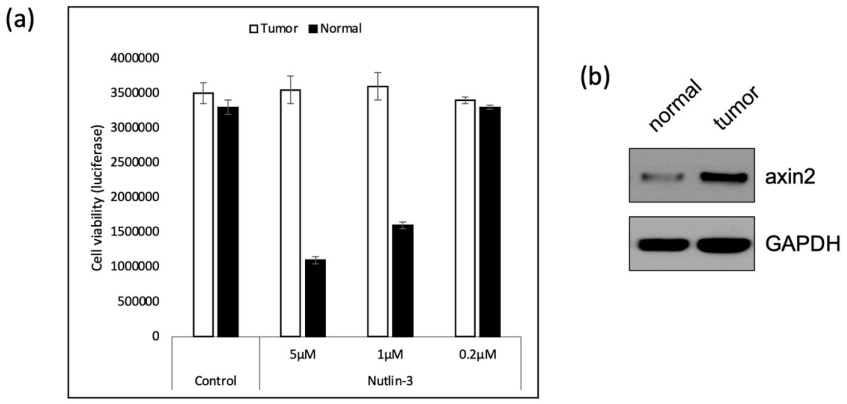

Fig. 3 Examining the genetic makeup of normal and tumor organoids
(a) Organoids from the tumor are insensitive to nutlin-3. Organoids were grown for one week with the indicated concentrations of nutlin-3. Organoid viability was assessed using luciferase assay.
(b) Western blot analysis of AXIN2 in normal and tumor organoid.

Grown medium constituents related to Wnt pathway	Working mechanism
WNT3a	Activation WNT signaling
R-spondin	WNT/B-catenin signaling antagonist
EGF	Epidermal growth factor
Noggin	Inhibition BMP signaling

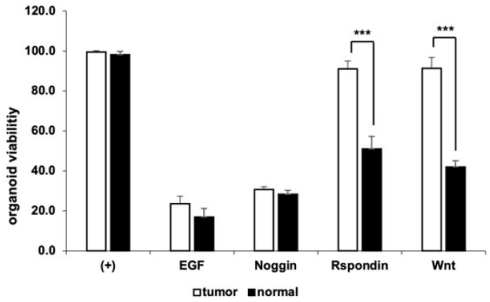

Fig. 4 Organoid viability under media constituents in normal and tumor organoids Frequently used growth media constituents, their working mechanisms and effects (left). Organoid viability was assessed using ATP.(***P<0.001)

in the APC and APC-related Wnt pathway. In conclusion, our findings suggest that while normal organoids possess wild-type APC and P53, tumor organoids harbor mutations in these genes.

Identification of The Minimal Set of Niche Factors Required for Culturing Organoid

In our study, we sought to identify the minimal set of niche factors required for the culture of organoids, which are essential for their growth. According to previous reports, microenvironment niche components of endogenous intestine stem cells allows for the supplementation of growth factors that constitute the niche signal[8]. For instance, this includes WNT (frizzled/LRP ligand) for stem cell expansion, noggin for stem cell maintenance, R-spondin (a WNT antagonist) for stem cell maintenance, and EGF for promoting cell proliferation[8]. Following the results shown in previous figures, to determine the minimal composition of niche factors based on the genetic background, we systematically removed each factor (WNT, EGF, noggin, R-spondin) from the culture media that initially contained all these factors. The viability of both tumor and normal organoids was then assessed through ATP evaluation after the withdrawal of each niche factor. The results revealed a significant decrease in cell viability for both tumor and normal organoids when EGF and Noggin were withdrawn, compared to the full media condition, with no significant difference in viability between tumor and normal organoids. Unexpectedly, the withdrawal of WNT actually led to an increase in viability in tumor organoids, while the withdrawal of R-spondin showed no significant change in viability. However, as expected, the withdrawal of WNT and R-spondin in normal organoids resulted in decreased viability. Thus, we were able to identify the minimal set of niche components required for the culture of tumor and normal organoids, especially establishing conditions for the withdrawal of WNT and R-spondin in tumor organoid cultures. This finding poses a potential for significantly reducing the time and effort required for organoid culture.

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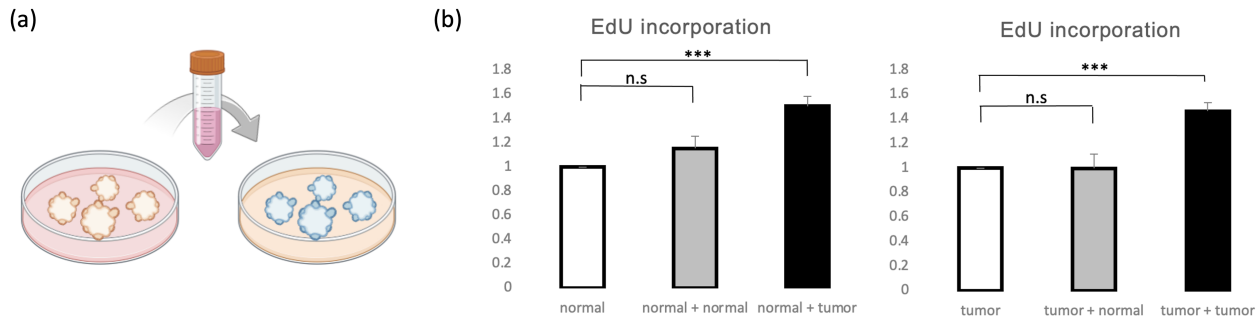

Fig. 5 Characterization of the features of cultured tumor organoids in medium
(a) Schematic diagram illustrating the transfer of tumor organoid cultured media to normal organoid
(b) The EdU incorporation rate (the ratio of EdU-positive normal and tumor organoid to total Hoechst 33342-positive normal and tumor organoid) is shown. (n.s; non- significant, ***P<0.001)

Identification of The Characteristics of Tumor Organoid Cultured Media

In our study, we next sought to understand the characteristics of tumor organoid cultured media. We observed that tumor organoids grew faster in vitro than normal organoids and questioned the cause of this difference. We hypothesized that an organoid-derived factor X, released during the culture of tumor organoids, could regulate the microenvironment, such as pH and oxygen levels, thereby influencing cell growth and differentiation. To test this hypothesis, we cultured normal and tumor organoids with media that had previously cultured either tumor or normal organoids. As a result, we observed only a small increase in growth in normal organoids cultured in the medium of normal organoids (normal + normal medium) and tumor organoids cultured in the medium of normal organoids (tumor + normal medium). However, we observed an increase in growth in normal organoids cultured with media from tumor organoids (normal + tumor media) and in tumor organoids cultured with their own media (tumor + tumor media). These findings confirmed that tumor organoid-derived factor X could indeed increase the growth of organoids.

Investigation of Tumorigenesis Through EMT By Tumor Derived Factor

In our research, we sought to determine the cause behind the observed increase in growth of normal organoids following the addition of tumor organoid-derived media (normal + tumor media). Based on previous findings, we hypothesized that tumor organoid-derived factor X could be associated with tumorigenesis. The process of tumorigenesis often involves EMT, prompting us to examine the expression of EMT-related markers in the normal organoids that exhibited increased growth. We found that the mRNA expression of markers indicative of the epithelial state, such as E-cadherin (CDH1), Fibroblast Growth Factor Binding Protein 1 (FGBP1), and Microphthalmia-Associated Transcription Factor (MITF),[5] decreased in normal organoids cultured with tumor media compared to those in normal media. Conversely, markers representative of the mesenchymal state, including Vimentin and N-cadherin, showed increased RNA expression. This suggests that the mere addition of tumor organoid cultured media to normal organoids can induce tumorigenesis. Consequently, we could demonstrate that factor X, present in tumor organoid cultured media, has the capacity to induce tumorigenesis.

Identification of Tumorigenesis by The Activation of mTOR Pathway

In our investigation, it was previously unknown how tumor-derived factor X could induce tumorigenesis through the regulation of specific pathways. There are many pathways that are activated in tumors, but we have seen that the presence of tumor-derived factor X can promote tumorigenesis in fig 6. We looked at the different pathways that promote tumorigenesis and found references that when the mTOR pathway is activated, it promotes tumorigenesis[9]. So we assumed that the tumor-derived factor promotes tumorigenesis through the mTOR pathway and proceeded the following experiment to prove this assumption. The mTOR pathway plays a critical role in various cellular processes including cell growth, metabolism, and survival[9]. To test our hypothesis, we examined the changes in proteins involved in the mTOR pathway in normal organoids and those cultured with tumor-derived media. The results showed an increase in the phosphorylation of mTOR and Akt, indicating the activation of the mTOR pathway in normal organoids exposed to tumor-derived media. This finding suggests that the mTOR pathway's activation by tumor-derived factor X could be a mechanism through which tumorigenesis is induced.

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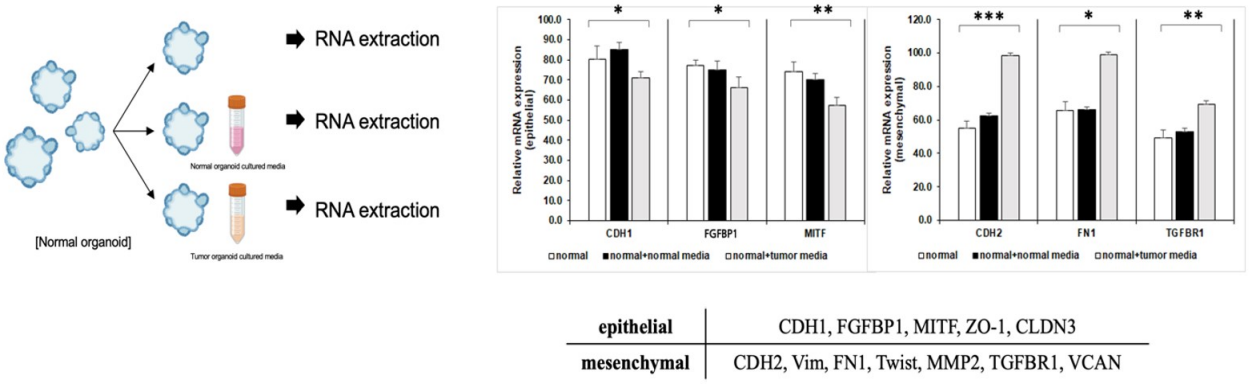

Fig. 6 Gene expressions in normal, normal + normal media and normal + tumor media organoids.
RT-qPCR analysis of the expression level of CDH1, FGFBP1, MITF, CDH2, FN1 and TGFBR1 in normal, normal+normal media and normal + tumor media.(*P<0.05, **P<0.01,***P<0.001).

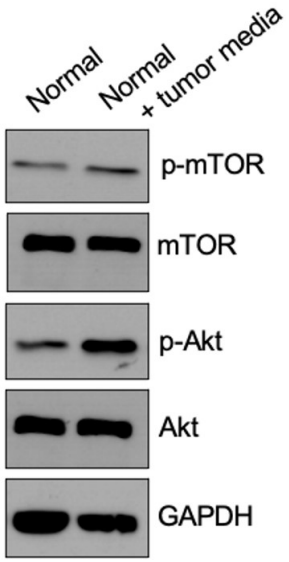

Fig. 7 Tumor-derived factor X can regulates mTOR pathway.
Western blot analysis performed to detect the protein expression levels of p-mTOR, mTOR, p-Akt, Akt and GAPDH. GAPDH

Discussion

Organoids are among the most physiologically relevant models available for studying the differentiation process of stem cells in a variety of regulated environments. To date, organoids have been proven to be an innovative experimental method for elucidating the unique characteristics (identity) of stem cells and the components of their surrounding microenvironment (niche components)[8].

In numerous studies, organoids have been used in conjunction with genetic information, transcriptomic, and proteomic analyses to uncover critical aspects of tissue development, homeostasis maintenance, and disease. The process of forming organoids that accurately replicate the composition of human tissues in vivo requires complex cultured media. We initially obtained normal and tumor organoids derived from a colon cancer patient to observe changes due to differences in their genetic features. These organoids exhibited differences in morphology but shared the common characteristic of possessing epithelial cell types.

Next, to verify if the colon cancer organoids reflect the genetic background of colon cancer, we confirmed the presence of mutations in P53 and APC through luciferase assays and western blotting. In the case of colon cancer-derived tumor organoids, a mutation in APC results in the constant activation of WNT signaling. Therefore, it is known that normal organoids always require WNT and R-spondin as niche factors, whereas tumor organoids do not. Unexpectedly, contrary to expectations, the viability of tumor organoids actually decreased in the presence of WNT. This suggests that excessive WNT supply in the context of an APC mutation could destabilize the survival mechanisms of the organoid.

Moreover, we were curious about the source of the growth difference between normal and tumor organoids, noting that tumor organoid growth was relatively faster. This led us to hypothesize the existence of a growth-promoting factor that could be released into the media. Consistent with our hypothesis, we discovered that adding tumor organoid cultured media to both normal and tumor organoids stimulated organoid growth.

EMT is a process where epithelial cells lose their cell polarity and cell-cell adhesion characteristics, gaining the ability to move or become invasive, transforming into mesenchymal cells. This process commonly occurs during tumorigenesis. We hypothesized that tumor-derived factor X could be involved in tumorigenesis and, upon examining EMT markers, we found a surprising result. In normal organoids to which tumor media was added, there was a decrease in the expression of epithe-

lial markers and an increase in the expression of mesenchymal markers.

In a previous study, the mTOR pathway was known to regulate various cellular functions including growth and proliferation as a kinase. Activation of the mTOR pathway is one of the most frequently occurring changes in human cancers, suggesting that inhibiting the mTOR pathway could be an effective anticancer strategy[9]. This prior finding supports the idea that the increased growth of organoids during tumorigenesis is due to the activation of the mTOR pathway. Consequently, upon examination, we observed the upregulation of proteins related to the mTOR pathway in normal organoids cultured with tumor media, indicating tumorigenesis.

In conclusion, we were able to ascertain that the phenomenon of tumorigenesis induced by tumor-derived factor X originates from the activation of the mTOR pathway. We know from multiple references that Factor X is extracellular vesicles (EVs), which are spherical double-membrane vesicles that are released into body fluids. EVs contain DNA, RNA, and proteins and are known to play an important role in cancer cell progression, metastasis, and chemotherapy resistance. The presence of EVs derived from tumor organoids can be one of the evidences of tumor microenvironment[10].

We have confirmed that factor X (EV) derived from tumor organoids can promote tumorigenesis by contributing to the mTOR pathway, but since tumorigenesis is a multi-step process in which cancer cells need to adapt to various microenvironments, it will be necessary to further study it in fibroblasts or immune cells. It will be the direction of our further research to confirm tumorigenesis by culturing fibroblasts and immune cells with tumor derived factor X.

Overall, this study may provide a new guideline for culture methods by presenting an economical culture method for normal and tumor organoids. In addition, we propose the possibility that tumor-derived factor X is extracellular vesicles and suggest that its presence drives tumorigenesis, which may be the first report to suggest that tumorigenesis proceeds through the mTOR pathway.

Materials & Methods

Abbreviation Table

CRC	colorectal cancer cell
mTOR	mammalian target of the rapamycin
EMT	epithelial to mesenchymal transtition
TME	tumor microenvironment
Wnt	winglessOrelated integration site
EV	extracellular vesicles

Organoid Culture

Organoids of cancerous and normal cells were procured from the Korean Cell Line Bank. WNT-conditioned medium (50%, produced using stably transfected L cells), B27 (Invitrogen), high-grade DMEM/F12 medium with nicotinamide (Sigma-Aldrich), N-acetylcysteine (Sigma-Aldrich), noggin (Peprotech), R-spondin (Invitrogen), EGF (Peprotech), and P38 inhibitor SB202190 (Sigma-Aldrich) were among the culture media. Organoids were grown in the presence of 5–10 μM neurotin-3 (Cayman Chemical) in order to select mutant P53. Mycoplasma contamination of organoids was routinely tested for, however the results were negative.

RNA isolation, cDNA preparation, and qRT-PCR

Organoids were collected in RLT lysis buffer in accordance with the manufacturer's instructions, and RNA was extracted using the Qiagen RNeasy kit (Qiagen). Extracted RNA was used as a template for the production of cDNA using GoScript reverse transcriptase (Promega), in compliance with the manufacturer's instructions. For qRT-PCR, IQ SYBR green mix (Bio-Rad) was utilized in compliance with the manufacturer's recommendations. The data were calculated using the $\Delta\Delta$ Ct approach.

Western blot analysis

The samples were lysed using 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Na-Deoxycholate, and 1% NP-40 RIPA buffer, to which complete protease inhibitors (Roche) were added. The protein content was measured using the Bradford test (BioRad), which is a common procedure. After running equal quantities of protein on SDS-PAGE gels, the protein was transferred to Millipore PVDF membranes. For APC Western blotting, protein lysates were loaded and spread out onto a gradient polyacrylamide gel (4–15%, BioRad). The membrane was blocked and probed with antibodies against Axin2(Abcam, ab109307), Akt/p-Akt(Cst, 9272), mTOR/p-mTOR(Cst, 5536) and GAPDH(santa cruz, sc-47724).

Organoid viability assay

Organoids were collected and filtered through a 40μm cell strainer (Falcon, USA) to eliminate any large organoids after two to three days of passaging. Then, organoids were put to ultralow-attachment 96-well plates (Corning, USA) after being reconstituted in 5% BME/organoid culture mix (15,000–20,000 organoids/ml). The organoids were given one microgram of Dox or vehicle controls. After three or five days of Dox incubation, cell viability was measured using CellTiter-Glo (Promega, USA) in accordance with the manufacturer's instructions. The data were then normalized to vehicle controls.

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EdU incorporation assay

By using the EdU (5-ethynyl-20-deoxyruidine) assay and the EdU assay kit (KeyGen Biotech., Nanjing, China), the proliferation of organoids was also observed. 24-well plates were seeded with organoids, and the plates were then incubated for 48 hours at 37 °C and 5% CO2. Following a 4-hour treatment with 10 mM EdU, organoids were fixed with 4% paraformaldehyde. After that, organoids were dyed using the combination solution of Click-iT EdU. Then DAPI was used to label the nuclei of the organoids.In accordance with the manufacturer's instructions, proliferation was measured as the proportion of cells actively integrating EdU in a microscopic field. Photographs were captured with a Zeiss microscope.

Statistical analysis

Each experiment was run at least three times in triplicate, and depending on the sample size, the results were shown as means ± SD. The figure legends provide information on other data format-related aspects as well as the statistical test that was used for each experiment. A difference was considered statistically significant if its P value was less than 0.05. Prior to each experiment, animal randomization was carried out for the in vivo investigations.

Table 1. mRNA primers

epithelial marker	forward(5'->3')	reverse(5'->3')
EPCAM	CAGAAGGAGATCACAACGCG	TCCAGATCCAGTTGTTCCCC
CDH1	CGGACGATGATGTGAACACC	TTGCTGTTGTGCTTAACCCC
ACTA2	ACCCAGCACCATGAAGATCA	AGAGACAGAGAGGAGCAGGA
VIM	GAGTCCACTGAGTACCGGAG	ACGAGCCATTTCCTCCTTCA
FGFBP1	GGAACACAAAGCCCAGGAAG	AGAACTCCAGGGCAGTCTTC
MITF	CCGTCTCTCACTGGATTGGT	GTTTGGACATGGCAAGCTCA
CDH2	CGGTTTCATTTGAGGGCACA	TTGGAGCCTGAGACACGATT
FN1	CCCCATTCCAGGACACTTCT	TGCCTCCACTATGACGTTGT
TGFBR1	AGCTTTGCCTGAACTCTCCT	GCTGTTTCCTGGGTCCAAAG

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References

1 L. Torre, R. Siegel, E. Ward and A. Jemal, *Cancer Epidemiol Biomarkers Prev*, **25**, 16–27.

2 T. Seidlitz, S. Merker, A. Rothe, F. Zakrzewski, C. Neubeck, K. Grützmann, U. Sommer, C. Schweitzer, S. Schölch, H. Uhlemann, A. Gaebler, K. Werner, M. Krause, G. Baretton, T. Welsch, B. Koo, D. Aust, B. Klink, J. Weitz and D. Stange, *Gut*, **68**, 207–217.

3 C. Pasch, P. Favreau, A. Yueh, C. Babiarz, A. Gillette, J. Sharick, M. Karim, K. Nickel, A. DeZeeuw, C. Sprackling, P. Emmerich, R. DeStefanis, R. Pitera, S. Payne, D. Korkos, L. Clipson, C. Walsh, D. Miller, E. Carchman, M. Burkard, K. Lemmon, K. Matkowskyj, M. Newton, I. Ong, M. Bassetti, R. Kimple, M. Skala and D. Deming, *Clin Cancer Res*, **25**, 5376–5387.

4 T. Xia, W. Du, X. Chen and Y. Zhang, *J Cell Mol Med*, **25**, 5829–5841.

5 P. Kakni, C. Lopez-Iglesias, R. Truckenmuller, P. Habibovic and S. Giselbrecht, *Front Bioeng Biotechnol*, **10**, 879024.

6 J. Drost, R. Jaarsveld, B. Ponsioen, C. Zimberlin, R. Boxtel, A. Buijs, N. Sachs, R. Overmeer, G. Offerhaus, H. Begthel, J. Korving, M. Wetering, G. Schwank, M. Logtenberg, E. Cuppen, H. Snippert, J. Medema, G. Kops and H. Clevers, *Nature*, **521**, 43–47.

7 C. Tovar, J. Rosinski, Z. Filipovic, B. Higgins, K. Kolinsky, H. Hilton, X. Zhao, B. Vu, W. Qing, K. Packman, O. Myklebost, D. Heimbrook and L. Vassilev, *Proc Natl Acad Sci U S A*, **103**, 1888–1893.

8 F. Holmberg, J. Seidelin, X. Yin, B. Mead, Z. Tong, Y. Li, J. Karp and O. Nielsen, *EMBO Mol Med*, **9**, 558–570.

9 G. He, L. Lin, J. DeMartino, X. Zheng, N. Staliarova, T. Dayton, H. Begthel, W. Wetering, E. Bodewes, J. Zon, S. Tans, C. Lopez-Iglesias, P. Peters, W. Wu, D. Kotlarz, C. Klein, T. Margaritis, F. Holstege and H. Clevers, *Cell Stem Cell*, **29**, 1333–1345 1336.

10 Y. Liao, Q. Yi, J. He, D. Huang, J. Xiong, W. Sun and W. Sun, *Bioengineered*, **14**, 113–128.