

Growth pattern of de novo small clusters of colorectal cancer is regulated by Notch signaling at detachment

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Abstract

Cancer cell clusters have a higher capacity for metastasis than single cells, suggesting cancer cell clusters have biological properties different from those of single cells. The nature of de novo cancer cell clusters that are newly formed from tumor masses is largely unknown. Herein, we generated small cell clusters from colorectal cancer organoids and tracked the growth patterns of the clusters up to four cells. Growth patterns were classified into actively growing and poorly growing spheroids (PG). Notch signaling was robustly activated in small clusters immediately after dissociation, and Notch signaling inhibition markedly increased the proportion of PG spheroids. Only a limited number of PG spheroids grew under growth-permissive conditions in vitro, but xenograft tumors derived from Notch inhibited clusters showed growth rates comparable to those of untreated spheroids. Thus, de novo clusters are composed of cells with interchangeable growth fates, which are regulated in a context-dependent manner by Notch signaling.

KEYWORDS

cancer cell cluster, cell fate, circulating tumor cell, inactive growth, Notch

Abbreviations: 5-FU, 5-fluorouracil; AG, actively growing; CRC, colorectal cancer; CTC, circulating tumor cell; CTOS, cancer tissue-originated spheroid; D, dead; GSEA, gene set enrichment analysis; NICD, Notch intracellular domain; PG, poorly growing; PI, propidium iodide; SFG, sphere-formation and growth.

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1 | INTRODUCTION

Cancer invasion and metastasis are major causes of cancer-related morbidity and mortality. Single cells have long been studied as units of invasion and metastasis. However, collective migration (the migration of cells as cohesive groups rather than as individual single cells) has been proposed and is now well recognized as a mode of invasion.¹ In addition, circulating tumor cell (CTC) clusters have been reported to be more capable of forming metastases than single cells in various cancers,²⁻⁴ including colorectal cancer (CRC).⁵ Using a liver metastasis model of CRC organoids, Okuyama et al. observed the process of CTC cluster implantation and metastasis formation in the liver, and showed that the clusters consistently maintain a cellular mass during the process of clearing vascular endothelium at the implantation site and establishing invasive and metastatic foci as a mass.⁶ This has also been confirmed in a peritoneal metastasis model of ovarian cancer.⁷ This suggests that implantation by de novo clusters is followed by the formation of metastases through collective migration and proliferation of cells as a mass. These findings suggest that clusters of cancer cells have biological characteristics that are different from those of single cells as cancer units. CTC clusters may be generated from tumors exposed to blood/lymphatic vessels or body cavities by the aggregation of single cells or direct cluster detachment (de novo clusters). Considering that cancer cells strongly induce cell death when dissociated into single cells⁸ and that single-cell viability in CTCs is low,⁹ de novo clusters may be a better source of CTC clusters than aggregates of single cells in the circulation; however, little is known about the characteristics of de novo CTC clusters.

Three-dimensional cultures of cancer cells from tumors have recently advanced through innovative organoid culture methods.¹⁰ Previously, we developed a method for preparing and culturing organoids from cancer tissues using the cancer tissue-originated spheroid (CTOS) method.⁸ Unlike organoids derived from single cells, organoids prepared by the CTOS method (CTOS organoids) are not dissociated into single cells during preparation or culture. Thus, this is an excellent platform for studying the characteristics of cancer cells as cell masses.

In our previous report, we showed that the mechanical disruption and reconstruction of CTOS organoids enhanced the proliferative potential and stemness of CRC,¹¹ suggesting that cancer cells within CTOS organoids can alter their proliferative potential by disrupting their 3D structures. This is similar to the wound-induced epithelial cell transition from non-stem to stem cells during normal intestinal wound healing.¹² Thus, the disruption of 3D cancer cell structures may alter the cell fate.

Colorectal cancer cells comprise distinct but interchangeable subpopulations with different growth rates.¹³ Tracking the fate of spheroid growth from single cells revealed that the growing CRC cells were either highly proliferative (L cells) or less proliferative (S cells). While L cell-derived spheroids are composed of both L and S cells, S cells maintain only S cell characteristics once isolated. S

cells can be converted to L cells by various stimuli, including Notch-mediated cell-cell interactions. Because the previous study focused only on actively growing subpopulations, little is known about the characteristics of subpopulations that are not growing or very slowly.

As Notch is a transmembrane receptor that plays a key role in local intercellular communication,¹⁴ it may also be involved in determining the nature of de novo cancer clusters. Notch has multifaceted functions in development and diseases, including cancer. Notch signaling functions differently in different cellular contexts. With different activation levels, timings, durations of expression, spatial distributions of activity, and cellular contexts, Notch can play diverse roles in a variety of processes, including cell fate determination, differentiation, proliferation, self-renewal, and apoptosis.¹⁵ Due to the importance and complexity of Notch signaling, its output must be carefully studied in the context of individual cells.

In this study, we focused on growth “fate” rather than “state”. Growth fate is the growth pattern several weeks after the point of observation, while state is the condition of the cell at the point of observation. We developed a model in which small cancer cell clusters detach from larger masses and characterized the “de novo clusters” of colon cancer cells. Using a modified spheroid formation assay to evaluate the growth patterns (fates) of small clusters dissociated from CRC CTOS organoids, we found that de novo clusters have a survival advantage over single cells, there are three types of cluster growth patterns, and Notch signaling plays a key role in determining growth patterns in a cellular context-dependent manner.

2 | MATERIALS AND METHODS

2.1 | Terminology

“CTOS organoids” are 3D cultured cancer cells prepared and passaged by the CTOS method. A “cluster” is a group of several cancer cells newly generated from the main tumors or large CTOS organoids. A “spheroid” is a spherical cancer cell mass monitored for growth from a single cell or clusters derived from CTOS organoids, and an “SFG spheroid” is a spheroid in the SFG assay described below.

2.2 | Fractionation of clusters and single cells

A filter (CELLNETTA; Murata Manufacturing) with a high opening ratio and precise pore size was used to fractionate clusters and single cells (Figures S1–S7). The CTOS organoids were dissociated and filtered through a 20 μ m filter. They were then filtered through a 6 μ m filter. The upper portion of the 20 μ m filter was a fraction containing small cell clusters, whereas the flow-through of the 6 μ m filter was a fraction comprising pure single cells. Propidium iodide (P3566; Thermo Fisher Scientific) and Hoechst 33342 (H3570; Thermo Fisher Scientific) staining of the cells were carried out at final concentrations of 1.5 μ M and 16.2 μ M, respectively.

2.3 | Sphere-formation and growth assay

The CTOS organoids were dissociated into nearly single cells by treatment with 0.25% Trypsin-EDTA (25200072; Thermo Fisher Scientific) for 8 min at 37°C, and all of the cells and clusters were then mildly pipetted with DNase I (10 µg/mL; Roche) for 1 min at room temperature (25°C). Single cells and cell clusters were counted, diluted, and plated on 384 round-bottom plates (MS-9384 U; Sumitomo Bakelite) to create one cell or one cluster/well in suspension condition. After the plates were allowed to stand for 2 h, microscopic images of all wells were obtained using a microscope (DMI4000B; Leica Microsystems) equipped with an automatically controlled XY stage (Mitani Corporation) to confirm the exact number of cells in each well. Wells containing single cells or two clusters were analyzed. In rare cases, wells containing multiple single cells were included in the clusters because they aggregated and formed clusters within 24 h of plating. Bright-field images were taken using the above-mentioned microscope, and the area of the SFG spheroids was measured using ImageJ Fiji software (<https://imagej.net/software/fiji>). The classification of the subpopulations with different growth patterns is described in the text.

Materials and methods for patient samples (Tables S1 and S2), CTOS organoid preparation and culture, Notch inhibitor treatment, immunostaining, generation of C45/Fucci, drug sensitivity test, western blot analysis, RT-PCR (Table S3), assays for mitochondria; RNA sequencing and bioinformatic analysis, xenotransplantation of cell clusters, and statistical analysis are described in Data S1.

3 | RESULTS

3.1 | Distinct growth patterns of newly generated single cells or clusters from CRC CTOS organoids

To examine the growth pattern of de novo clusters of CRC cells, CTOS organoids from C45, a patient-derived CRC CTOS organoid line, were subjected to SFG assay (see Materials and Methods). Spheroid-forming capacity increased along the initial cell number, and the effect saturated at four to five cells (Figure S2A). It is reported that the majority of CTC clusters were two to five cells in non-small-cell lung cancer patients.¹⁶ Using mathematical estimation, the number of CRC cells in a CTC cluster during metastasis in vivo was estimated to be two to three in the liver.³ Based on these results and information, the number of cells in de novo clusters in this study was set to two to four. After accurately counting the number of cells in each well, the growth of each single cell or small cell cluster (two to four cells) was assessed (Figure 1A–C). Some single cells or clusters grew to form SFG spheroids, whereas others died immediately after plating (Figure 1C). The growth potential of each cell or cluster varied considerably for C45. All SFG spheroids above 5000 µm² on day 14 grew continuously, while those below 5000 µm² did not grow at all, except for one that grew very slowly (Figure 1B). This growth pattern was observed in all four CRC CTOS organoids (C45, KUC3, KUC6, and KUC16) (Figure S2B), suggesting the existence of distinct

cell subpopulations with different growth potentials. The growth patterns were divided into three categories: actively growing (AG), poorly growing (PG), and dead (D). To discriminate AG from PG, a threshold of 5000 µm² by day 14 was set for C45 cells (Figures 1B and S2A), and by day 21 for the other three lines (Figure S2B) due to different growth rates. In Group D, the cells were fragmented into small pieces (Figure 1C). Spheroid-forming capacity was defined as the ratio of AG spheroids to the total plating of single cells. The clusters showed higher spheroid-forming capacities than the theoretical values calculated from single cells (this method is described in the legend of Figure S2C). In all four CTOS organoid lines, the ratio of the AG group increased with increasing initial cell numbers, that of the D group decreased, and that of the PG group remained unchanged (Figures 1D and S2D). The results showed that de novo clusters exhibited distinctly different growth fates and that the clusters were advantageous for cell survival after dissociation.

3.2 | Subpopulations of poorly growing SFG spheroids

Because of their unique growth patterns, we focused on PG spheroids. PG spheroids have not been well studied in conventional spheroid formation assays because PG spheroids and dead cells have been grouped together in the “nonspheroid-forming” fraction. When cell death was assessed by PI staining, a small number of PI-positive cells were observed outside the SFG spheroids in both the AG and PG groups, with no difference between the groups (Figure 2A). However, when proliferation was assessed by Ki-67 staining, the number of positive cells was higher in AG spheroids than in PG spheroids (Figure 2B). Real-time Fucci imaging¹⁷ was used, because the number of PG spheroids was too small for quantification. The ratio of green nuclei (S, G₂/M) was significantly higher in AG spheroids, and most PG spheroids contained pure red nuclei (G₁/G₀) (Figure 2C,D). As a small number of PG spheroids contained green nuclei (Figure 2D), it is possible that the PG group was composed of two subgroups: a majority of nongrowing spheroids and a few very slowly growing spheroids. To assess whether the PG spheroids became AG spheroids under more growth-permissive conditions, day-16 PG spheroids from the SFG assay were selected and cultured in suspension or 2% Matrigel for an additional 7 days. The PG spheroids (Figure 2E, left) barely grew in suspension from days 7 to 14 (Figure 2E, right). In Matrigel, most PG spheroids (Figure 2F, left) did not grow, with only a few growing SFG spheroids (Figure 2F, right). In three independent experiments, there was no significant difference in the frequency of expandable PGs between suspension (2/55) and Matrigel (6/80) (Fisher's exact test). The growth of three exceptional SFG spheroids (#10, #30, and #32) (Figure 2F, right) was tracked. First, the three SFG spheroids were expanded and subjected to a second round of SFG assay. All the SFG spheroids generated AG spheroids from the clusters (Figures 2G and S3). The ratios of the groups were similar to those of the parental C45 CTOS organoids (Figure 2G). The ability of the AG spheroids to grow was higher in the four #32 derived cells than in the parental C45 cells (Figure S3).

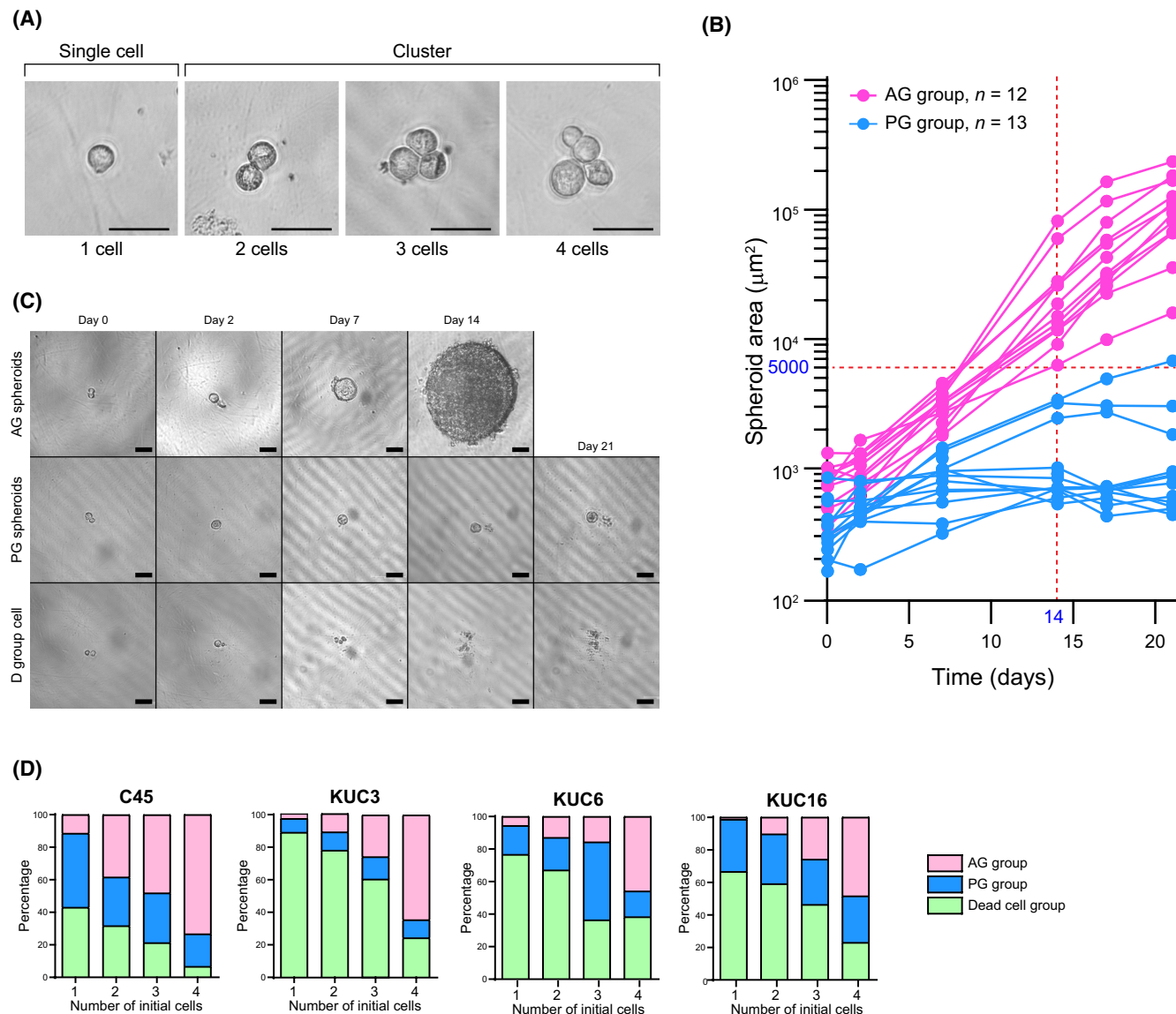


FIGURE 1 Analysis of the growth pattern of cancer cells derived from colorectal cancer tissue-originated spheroid organoids as single cells or clusters. (A) Representative microscopic images of C45 cells obtained using the sphere-formation and growth (SFG) assay. Single cells and clusters (two to four cells) are shown. Scale bar, 50 μm . (B) Growth curves of SFG spheroids derived from single cells or clusters of C45 cells using the SFG assay. SFG spheroids in the actively growing (AG) and poorly growing (PG) groups are indicated in pink and blue, respectively. The red dashed line indicates the threshold (5000 μm^2) at day 14 that discriminates between AG and PG spheroids. (C) Representative microscopic images of C45 SFG spheroids obtained using the SFG assay on the indicated days. An image of the AG spheroid on day 21 is not shown because of its poor viability due to overgrowth. Scale bar, 50 μm . (D) Stack graphs indicating the proportion of each group. D, dead.

Taken together, the difference between AG and PG spheroids occurred because of proliferation and not cell death, and the PG phenotype was stable in most PGs; however, a few exceptions gave rise to AG spheroids under growth-permissive conditions.

3.3 | Poorly growing spheroids were metabolically inactive

To further investigate the PG spheroids, the gene expression profiles of C45 PG and AG spheroids were compared using RNA

sequencing (Figure 3A). The GSEA results showed that senescence and TP53 pathway signatures were significantly enriched in PG spheroids (Figure 3B). Regarding senescence-related genes, *MAP2K3* and *THBS1* were upregulated (Figure 3A), whereas *CDKN2A* (p16) did not increase and *LMNB1* increased, suggesting that this was not typical of senescence.¹⁸ The expression of TP53-related genes, including *TP53*, *MDM2*, *RB1*, and *CDKN1A* increased (Figure 3A). Transcriptional upregulation of TP53 in the PG spheroids was confirmed by RT-PCR in three lines (C45, KUC3, and KUC6), but not in KUC16 (Figure S4A). The point mutation G151S of *TP53* in C45 (Table S2) might not be pathogenic; however,

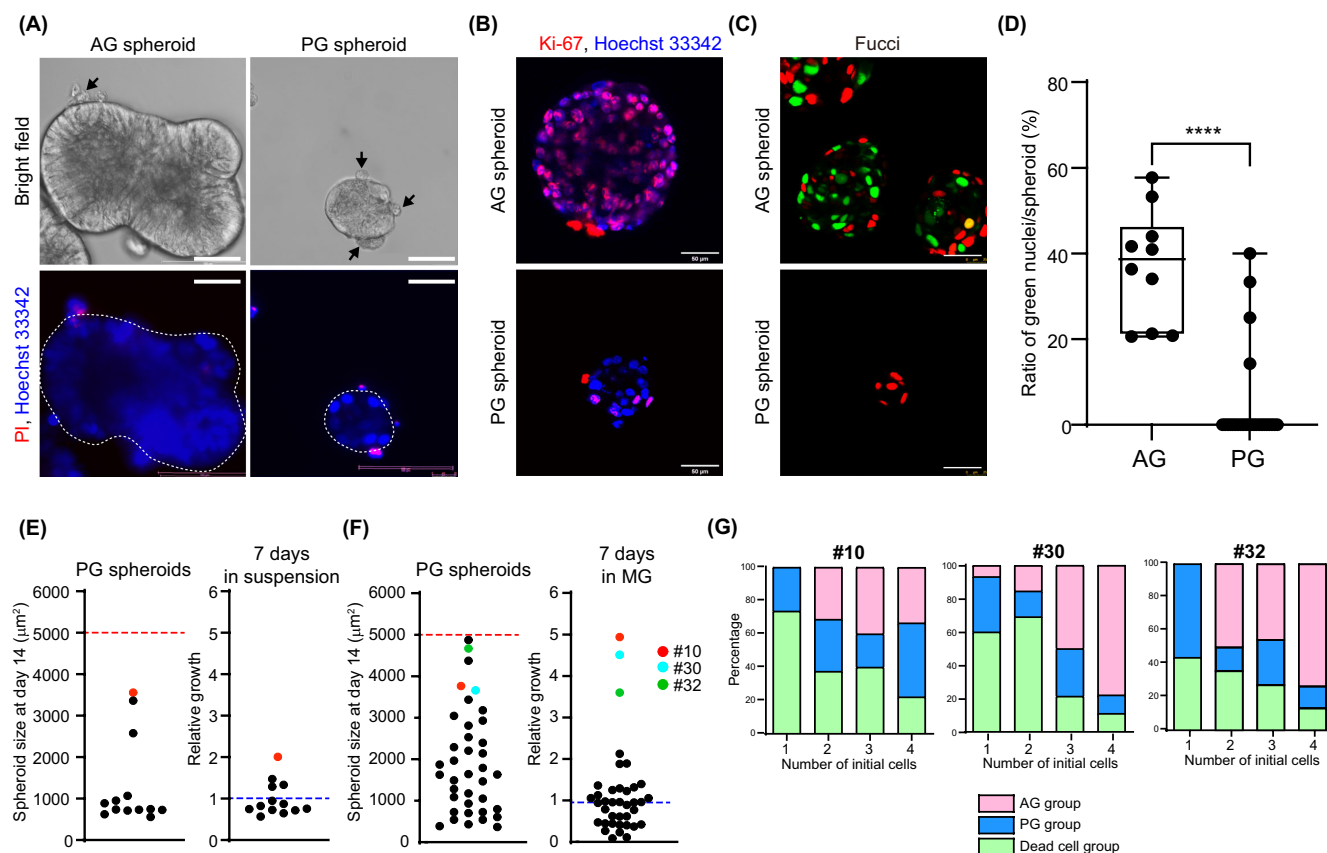


FIGURE 2 Proliferation and cell death in subgroups of sphere-formation and growth (SFG) spheroids in the SFG assay. (A–C) Representative immunofluorescent images of (A) propidium iodide (PI), (B) Ki-67, and (C) Fucci of C45 on day 16 of the SFG assay. PI-positive cells are indicated by arrows in the bright-field image in (A). Scale bar, 50 μ m. (D) Quantitative analysis of the Fucci images. The ratio of green nuclei to total nuclei (green + red) for each SFG spheroid is shown. Dots represent the ratio of each spheroid. (E, F) Scatterplots of the size of each poorly growing (PG) spheroid (C45) on day 14 of the SFG assay (left) and the relative growth of those SFG spheroids over another 7 days (right) under (E) suspension or (F) in 2% Matrigel (MG). Colored dots indicate the SFG spheroids that grew more than twofold (E, right; F, right). (G) Stack graph of the SFG assay of regrown SFG spheroids in (F). AG, actively growing. **** $p < 0.0001$.

V172F in KUC16 is reportedly pathogenic.¹⁹ Furthermore, KUC3 and KUC6 harbored truncating mutations at Arg342* and Arg196* of TP53, respectively, and the loss of the WT allele was confirmed by western blotting (Figure S4B). Thus, although TP53 transcripts were induced in PG spheroids, TP53 itself might not be essential for the PG phenotype. The clinical relevance of the PG features was analyzed using the GSE19860 dataset, which contained data corresponding to 29 CRC tumor samples from patients who had received chemotherapy (mFOLFOX6). Nine patients responded well to the treatment, whereas 20 did not. Interestingly, the top 100 genes upregulated in PG spheroids compared to AG spheroids (Table S4) were enriched in genes highly expressed in non-responders to chemotherapy, and the opposite was observed for downregulated genes (Figure 3C), implying that resistance to chemotherapy and the PG phenotype could have common underlying mechanisms. Indeed, PG spheroids, with the exception of KUC3, in which AG spheroids were resistant to 5-FU (Figure S4C). Gene ontology analysis revealed decreased expression of genes related to translation and mitochondrial respiration in PG spheroids (Figure 3D). Phospho-S6 immunostaining, a

marker of metabolic activity, was remarkably lower in PG spheroids²⁰ (Figure 3E). In addition, mitochondrial status was assessed using MitoTracker Green, which accumulates in the mitochondria in response to changes in mitochondrial membrane potential. PG spheroids were compared with size-matched parent C45 CTOS organoids. This is because the dye did not penetrate the core region of the larger CTOS organoids due to insufficient dye permeability.²¹ The MitoTracker intensity of the PG spheroids was significantly lower than that of the parent C45 CTOS organoids, with a few exceptions being compatible with the parental C45 CTOS organoids (Figure S4D,E). Taken together, these results indicated that translation and mitochondrial function were suppressed in PG spheroids.

3.4 | Notch signaling was activated in the de novo clusters dissociated from the CTOS organoids

We previously reported that Notch signaling is involved in determining the growth fate of single CRC cells in a cell-cell

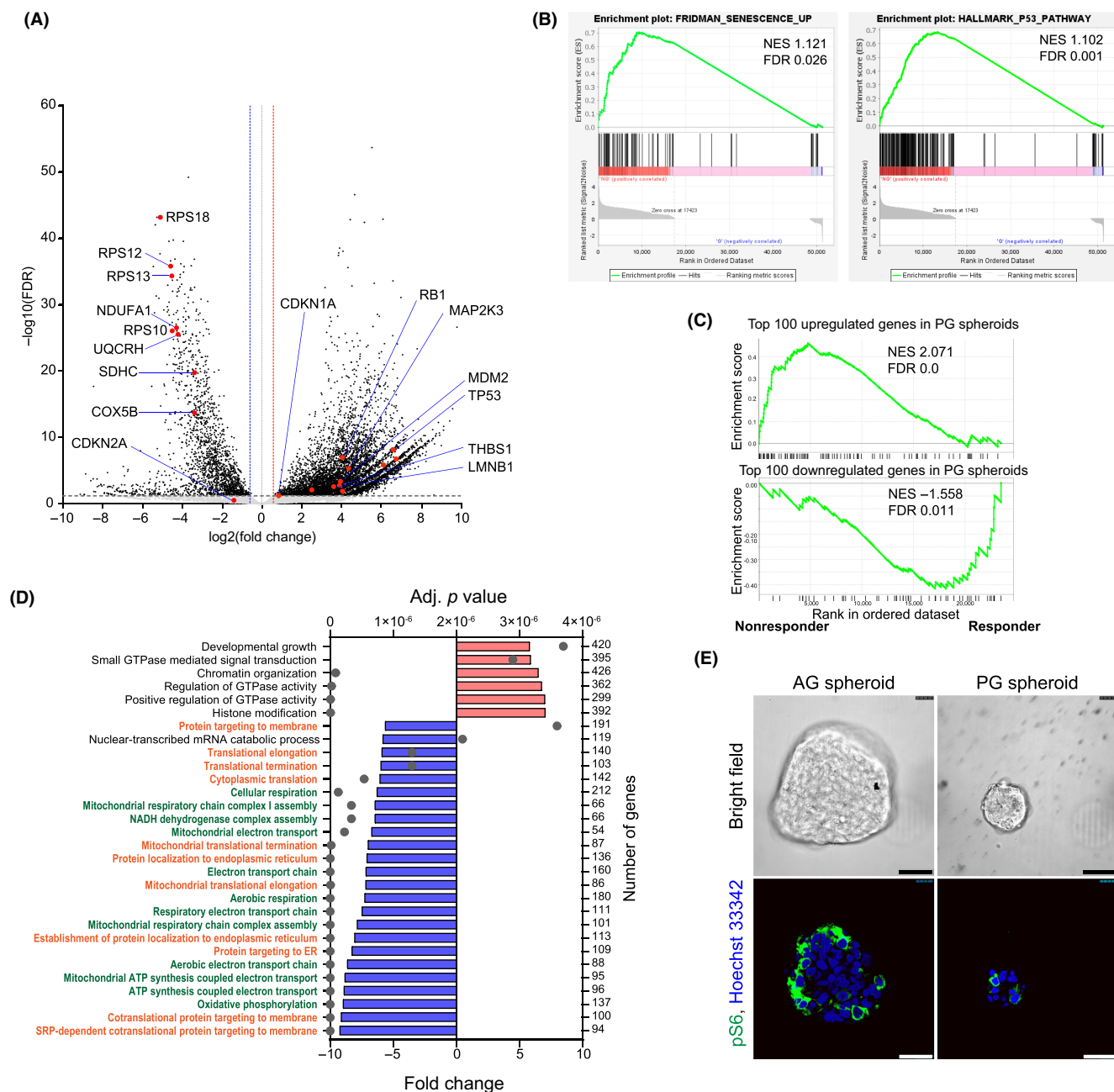


FIGURE 3 Gene expression, mitochondrial activity, and translational activity of poorly growing (PG) spheroids. (A) Volcano plots of differentially expressed genes comparing PG and actively growing (AG) spheroids in C45. Representative genes are indicated. The threshold lines are shown as ± 0.59 for $\log_2(\text{fold change})$ and 1.2 for $-\log_{10}(p \text{ value})$. (B) Gene set enrichment analysis (GSEA) comparing PG and AG spheroids using the senescence and TP53 gene sets. (C) GSEA plots depicting the relationship between genes in responders and nonresponders to chemotherapy (GSE19860). Genes are ranked by the statistical significance of differential expression analysis between the responders and nonresponders on the x-axis, with upregulated genes in nonresponders on the left side. The enrichment score of the top 100 up- or downregulated genes in the PG signature are plotted as green curves. (D) Gene ontology analysis comparing PG and AG spheroids. Orange letters indicate translation-related pathways; green letters indicate mitochondria-related pathways. (E) Representative microscopic images of bright-field (top panels) and immunofluorescence staining of pS6 (bottom panels). Green, pS6; blue, Hoechst 33342. Scale bar, 50 μm . FDR, false discovery rate; NES, normalized enrichment score.

contact-dependent manner, and that Notch activation stimulates the transition from S cells to L cells.¹³ We thus investigated the role of Notch signaling in de novo CRC clusters. Upon ligand binding, the Notch receptor is cleaved, releasing the NICD for downstream signaling.²² The NICD levels sharply increased immediately

after CTOS organoid dissociation in all four lines (Figure 4A), and the expression of downstream molecules of Notch, *HES1*, and *CDKN1A*, increased (Figure 4B). Notch signaling activation was then compared between single cells and small clusters. As Notch signaling changes rapidly after dissociation, fractions of single

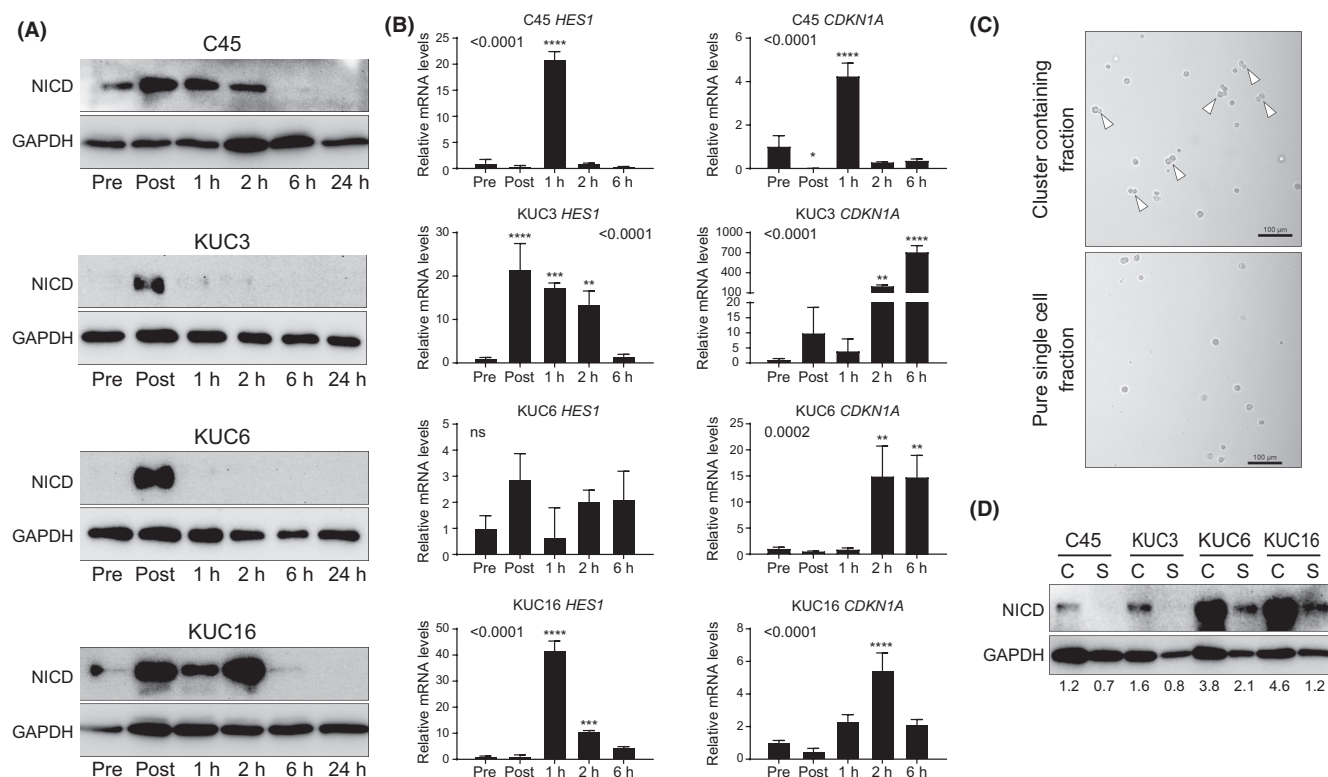


FIGURE 4 Activation of Notch signaling after cancer tissue-originated spheroid (CTOS) organoid dissociation. (A) Time course of Notch activation after CTOS organoid dissociation analyzed by western blot of cleaved Notch (NICD). Pre, before dissociation; Post, immediately after dissociation. (B) Time course of Notch activation after dissociation by RT-PCR of the indicated Notch downstream molecules. $n = 3$. Mean and SD is shown; p values for ANOVA tests are indicated when statistically significant. Individual significance by Tukey's test is indicated by asterisks. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (C) Representative images of the single cell and small cluster fractions prepared from C45 CTOS organoids. Small clusters are indicated by arrowheads. Scale bar, 100 μ m. (D) Western blot of the cleaved Notch (NICD) immediately after CTOS organoid dissociation. Values below indicate relative expression of NICD to GAPDH. C, cluster fraction; S, single cell fraction.

cells and clusters were prepared quickly and gently using filters with high opening ratios and precise pore sizes (Figures S1–S7). The fraction below the 6 μ m filter was of pure single cells, and between the 6 μ m and 20 μ m filters was a mixture of single cells and small cell clusters (Figure 4C). After dissociation, the NICD levels were much higher in the small cluster-containing fraction than in the single-cell fraction (Figure 4D). Thus, when CTOS organoids are dissociated, Notch signaling is activated in small clusters but not in single cells.

3.5 | Inhibition of Notch signaling increased the poorly growing group ratio in clusters

To investigate the functional role of Notch signaling in determining growth patterns in de novo clusters, Notch signaling was blocked using the γ -secretase inhibitor DAPT. The transient induction of NICD (Figure 5A) and the expression of its downstream molecules, HES1 and CDKN1A (Figure 5B), were significantly suppressed in all lines when DAPT was added to the medium, except for HES1 in KUC6. Notch inhibition in de novo clusters significantly increased the ratio in the PG group and decreased it in the AG group (Figures 5C

and S5A,B). This change was not observed in single cells. Therefore, Notch signaling may play different roles at different times. In addition, the effective timing of DAPT treatment to alter the growth patterns varied by line. For C45 and KUC6, DAPT treatment immediately after dissociation (early treatment) effectively changed the growth pattern (Figures 5C and S5A–C). However, no significant changes were observed when DAPT was added 1 day after dissociation (late treatment) (Figure S5A,C,D). In contrast, KUC3 and KUC16 increased the PG group in the delayed treatment (Figures 5C and S5A–C), whereas early treatment did not increase the PG group but increased the D group (Figure S5A,C,E). Thus, activated Notch signaling in de novo clusters is generally involved in avoiding the formation of PG spheroids but is also involved in anticell death in death-prone lines such as KUC3 and KUC16.

We further examined the effect of activating Notch signaling by a Notch ligand, JAG1. Western blotting revealed that addition of JAG1 at the formation of de novo clusters transiently increased the levels of NICD in all four lines (Figure S6A). Overall, addition of JAG1 did not change the ratio of AG, PG, and dead cells, with the exception of single cells and three cells of C45 where the ratio of the AG group significantly increased (Figure S6B). As the ratio of dead cells decreased in single cells, the increase in the ratio of AG group was due to the

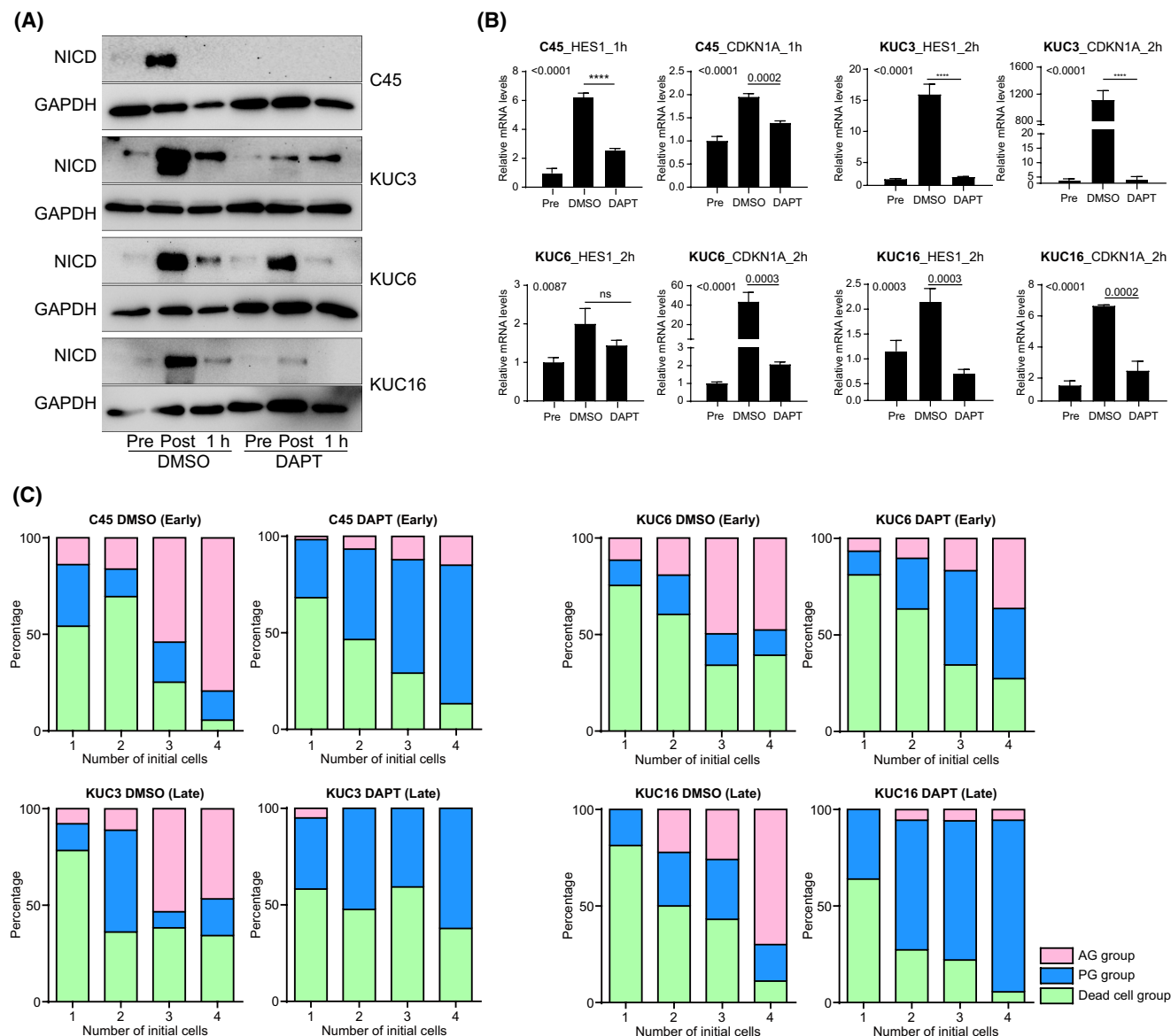


FIGURE 5 Effect of Notch inhibition on growth patterns in the sphere-formation and growth assay. (A) Western blot of Notch intracellular domain (NICD) with and without DAPT treatment; Pre, before cancer tissue-originated spheroid organoid dissociation; Post, immediately after dissociation. DAPT was added 1 h before dissociation. (B) RT-PCR of the indicated Notch downstream molecules with and without DAPT treatment. DAPT was added immediately after dissociation. *p* values for ANOVA tests are indicated when statistically significant. Individual significance by Tukey's test is indicated by asterisks. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. (C) Stack graphs showing the ratio of growth patterns on day 14 (C45) or 21 (KUC3, 6, 16). DAPT was added immediately after dissociation (Early) for C45 and KUC6 and 1 day after dissociation (Late) for KUC3 and KUC16. AG, actively growing; ns, not significant; PG, poorly growing.

avoidance of cell death by JAG1. In KUC6, the ratio of the AG group rather decreased by JAG1. These results indicate that Notch activation alone is not sufficient for the AG/PG transition and that factors that cooperate with Notch signaling are required. The level of Notch signal activation by dissociation is robust and may be saturated.

Notch activation is unlikely to be directly involved in SFG spheroid growth because late treatment with DAPT did not affect the ratio of the AG group in C45 and KUC6. In addition, DAPT treatment of larger CTOS organoids (40–100 μ m in diameter), used for drug sensitivity testing in the previous reports,^{23,24} showed no significant growth inhibition except for a marginal effect in KUC3 (Figure S7A).

DAPT treatment of smaller CTOS organoids (20–40 μ m in diameter) did not show growth inhibition at all (Figure S7B). These data indicate that Notch signaling is involved in determining cell survival and growth fate but not directly in growth itself.

3.6 | Notch inhibition-induced PG spheroids can grow in vivo

The SFG assay yields only a limited number of PG spheroids. To further investigate the diverse roles of Notch inhibition in cancer

cell clusters, a sufficient number of C45 clusters were collected on a 20 μm filter (Figures 6A and S1–S7). The clusters were cultured with or without DAPT. The sizes of all the spheroids derived from the clusters were evaluated on day 7 because the spheroids began to aggregate after 7 days of culture in the same dish. Similar

to the SFG assay, DAPT treatment increased the number of small spheroids (Figure 6B). To examine the regrowth potential after DAPT treatment, PG spheroids were collected after the SFG assay (Figure 6C left) and cultured for 7 days in 2% Matrigel. After transfer to Matrigel, most PG spheroids retained PG, but a few grew

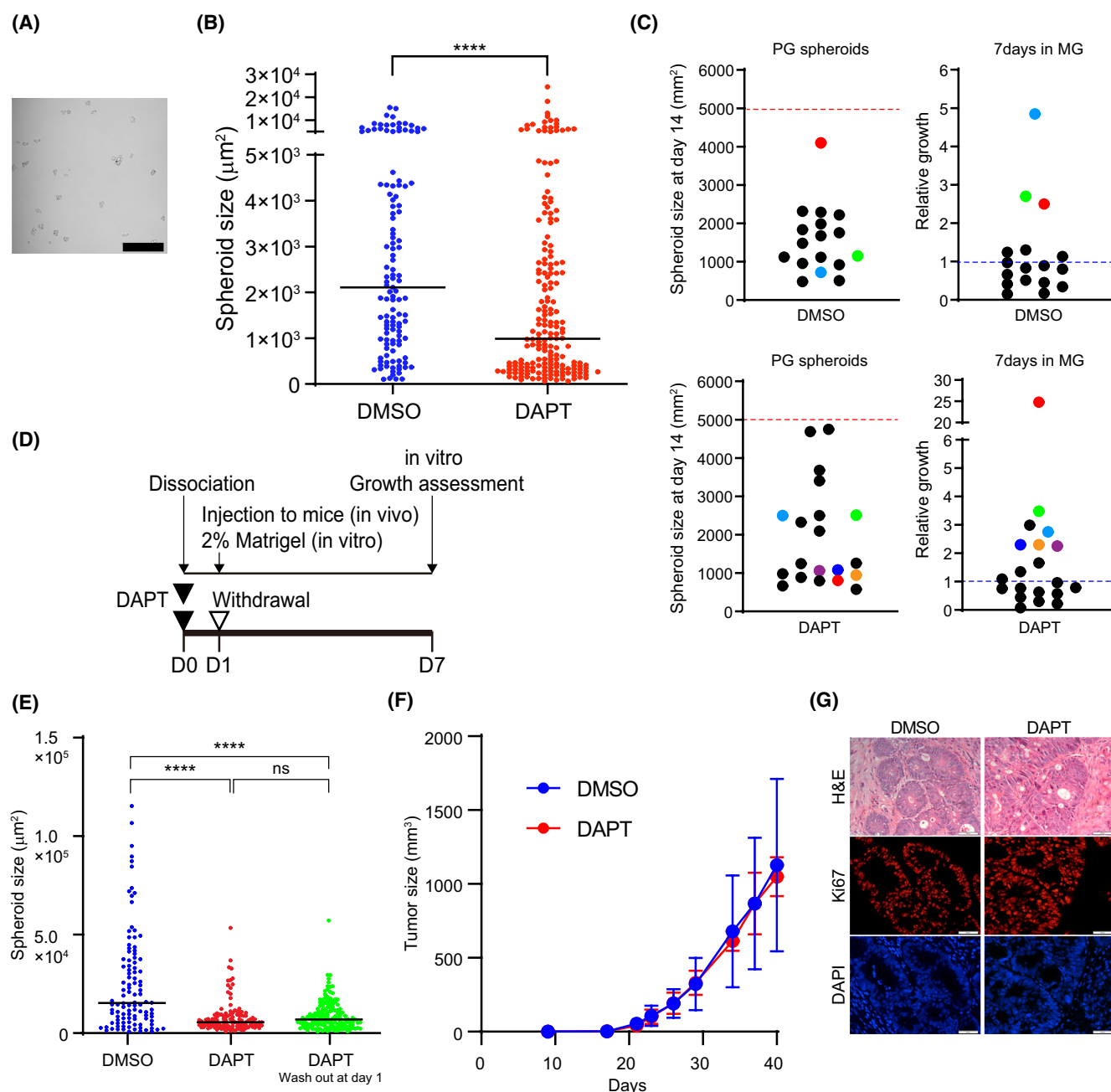


FIGURE 6 Effect of Notch inhibition on the growth potential of poorly growing (PG) spheroids in vitro and in vivo. (A) Representative image of small cell clusters of C45 over 20 μm filter immediately after preparation. Note that most single cells were removed. Scale bar, 250 μm . (B) Scatterplot of the sizes of each filter-prepared spheroid on day 7 (D7). Line indicates the median. p values for the Mann-Whitney U -test are indicated by asterisks. (C) Scatterplots of the size of each PG spheroid (C45) on day 14 of the sphere-formation and growth (SFG) assay (left) and the relative growth of those spheroids over another 7 days (right) in 2% Matrigel (MG). Colored dots indicate the spheroids that grew more than twofold in the right graphs. (D) Protocol for cell clusters showing timing of DAPT treatment, growth assessment, and subcutaneous injection into mice. (E) Scatterplots of the size of each spheroid derived from the clusters on day 7 cultured in 2% Matrigel according to the protocol in (D). Lines indicate the median. (F) Growth curves of tumors derived from small clusters injected subcutaneously on day 1 after DAPT or DMSO treatment. $n = 4$. Mean $\pm$ SD is shown. (G) Representative histology of the xenograft tumors in (F). Scale bar, 50 μm . **** $p < 0.0001$. ns, not significant.

by more than twofold (Figure 6C, right). Three independent experiments showed no significant difference in the frequency of expandable PGs between the DMSO (14/68) and DAPT (14/77) treatments (Fisher's exact test). Thus, the PGs that were increased by DAPT treatment were similar to the spontaneous PGs in that there were few expandable clones. Next, clusters were cultured in 2% Matrigel from day 1 to examine the effects of DAPT on growth (Figure 6D). Indeed, the clusters grew faster in Matrigel than in the suspension (Figure 6B,E). When the clusters were treated with DAPT at dissociation, most formed small spheroids, even when DAPT was removed on day 1 (Figure 6E). Thus, Notch inhibition immediately after dissociation plays a role in determining the subsequent growth patterns *in vitro*. Finally, we examined whether the slow-growth phenotype induced by DAPT treatment during dissociation was maintained *in vivo*. Small clusters were treated with or without DAPT for CTOS organoid dissociation and injected into the mice on day 1 (Figure 6D). In contrast to the slow-growing phenotype of the small spheroids *in vitro*, the DAPT-treated clusters formed tumors at the same rate as the untreated clusters *in vivo* (Figure 6F). In fact, the Ki-67 positivity of tumors derived from DAPT-treated clusters was compatible to that from DMSO-treated clusters (Figure 6G). It is possible that a small number of expandable PGs or contaminated AGs are involved in tumor growth; however, it is difficult to explain why they showed the same level of tumor growth as the *de novo* clusters that were not treated with DAPT. Thus, the fate determination of PG by Notch inhibition may be canceled *in vivo*.

4 | DISCUSSION

The finding that CRC cells comprise distinct subpopulations with different growth fates is consistent with the findings of our previous study.¹³ In this previous study, we tracked the growth fate of single cells under growth-permissive 2% basement membrane matrix conditions, and analyzed only AG subpopulations. Our present study focused on a novel subpopulation, the PG fraction, by tracking the growth fates of single cells and cell clusters under suspension conditions that mimic those in the bloodstream. The basic difference between S cells and PG cells is that S cells grow constantly, whereas PG cells mostly stop growing, with rare exceptions in which cells grow very slowly. Furthermore, a senescence signature was enriched in PG cells compared to that in AG cells, but not in S cells. Unlike S cells, most PG cells do not proliferate in Matrigel. Even larger spheroids in the PG fraction did not necessarily proliferate in Matrigel. Therefore, contamination of PG cells with S cells is rare, if any.

While the cell state can be easily altered by various factors in the microenvironment or cell-cell interactions, cell fate cannot be easily altered because of epigenetic regulation.²⁵ Notch signaling is activated upon dissociation into small clusters and the cells proliferate. When Notch signaling was inhibited, the cells did not proliferate. Notably, Notch signaling did not affect proliferation after

spheroid formation. Thus, Notch signaling does not directly control the cell state but rather determines its cell fate to proliferate; PGs are cells destined not to proliferate, and most PGs do not easily become AGs. Notch signaling plays a role in evading cell death through the AKT/PI3K and nuclear factor- κ B pathways.²⁶ This may be an effect of Notch signaling on the state of the cell. Notch signaling, on the other hand, plays an important role in cell fate decision.²⁷ The mechanism of lineage determination has been actively studied, but the regulation of somatic stem cells between active and quiescent states is not well understood. The PG phenotype is similar to that of quiescent stem cells. Notch inhibition with DAPT immediately after dissociation increased the PG fraction, but the window of the effect was quite narrow, indicating steady-state levels of Notch are not an issue. Notch signaling is reportedly high in the quiescent state and oscillatory in the active state.²⁸ Dynamic change in the Notch levels might be important in determining growth fate to AG. The mechanism by which only a few PG spheroids regrow under permissive growth conditions *in vitro* (Figure 2F) was not elucidated in this study. Heterogeneous activation of Notch signaling following dissociation into single cells or clusters may be a mechanism underlying the different growth rates.

In contrast to the difficulty of awakening PG spheroids *in vitro*, the growth rates of tumors derived from DAPT-treated and untreated cells were almost identical when injected subcutaneously into mice (Figure 6); the nongrowing fate determined by Notch inhibition *in vitro* was completely canceled by the special *in vivo* microenvironment. Several studies have proposed a role for ECM components in the awakening of quiescent cells,^{29,30} but Matrigel could not make the PG spheroids into AG spheroids. Growth factors and cytokines secreted by stromal cells, immune cells, and fibroblasts may also play a role.³¹ Factors that cooperate with Notch signaling for AG/PG transition may be elucidated in the future with the hint that PG cells easily transition to fast growing cells *in vivo*.

In our *de novo* cell cluster model, some *de novo* cell clusters and single cells grew into PG spheroids and were chemotherapy resistant. In addition, GSEA using a dataset of chemotherapy-treated patients showed that the gene expression profile in PG cells was associated with nonresponders to 5-FU. When CRC CTOS organoids were treated with anticancer drugs, the remaining intact cancer cells formed clusters, which were destined to be slow-growing cells.¹³ This suggests that persister cells after treatment of anticancer drugs resemble *de novo* clusters. Furthermore, the PG phenotype dramatically changed to the AG phenotype *in vivo*. These results indicate that cancer cells with the PG phenotype may evade therapy, regrow in the appropriate microenvironment, and cause recurrence. If PG-like fate is generated by treatment as CTC, targeting CTC clusters or PG-like cancer cells will be an important issue in future cancer treatment strategies.

AUTHOR CONTRIBUTIONS

Yi-Kai Lin: Formal analysis; investigation; methodology; visualization; writing – original draft. **Roberto Coppo:** Data curation;

formal analysis; supervision; writing – review and editing. **Kunishige Onuma**: Methodology; supervision; writing – review and editing. **Hiroko Endo**: Formal analysis; investigation; methodology; writing – review and editing. **Jumpei Kondo**: Supervision; writing – review and editing. **Sadahiro Iwabuchi**: Data curation; formal analysis; writing – review and editing. **Shinichi Hashimoto**: Data curation; formal analysis; supervision; writing – review and editing. **Yoshiro Itatani**: Resources; supervision; writing – review and editing. **Kazutaka Obama**: Resources; supervision; writing – review and editing. **Masahiro Inoue**: Conceptualization; formal analysis; funding acquisition; project administration; supervision; visualization; writing – original draft.

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CONFLICT OF INTEREST STATEMENT

J.K., K.O., and M.I. belong to the Department of Clinical Bioresource Research and Development at Kyoto University, which is sponsored by KBBM, Inc. M.I. is an inventor of the patents related to the CTOS method. The other authors have no conflict of interest for this article.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available in the DDBJ (accession number: DRA017130).

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: This study was approved by the Institutional Ethics Committees at Osaka International Cancer Institute (1803125402) and Kyoto University (R1575, R1671).

Informed consent: Fresh surgical specimens from CRC patients were obtained after obtaining written informed consent.

Registry and the registration no. of the study/trial: N/A.

Animal studies: All animal experiments were approved by the Institutional Animal Care and Use Committee of Kyoto University (18564). All procedures were performed in compliance with institutional guidelines.

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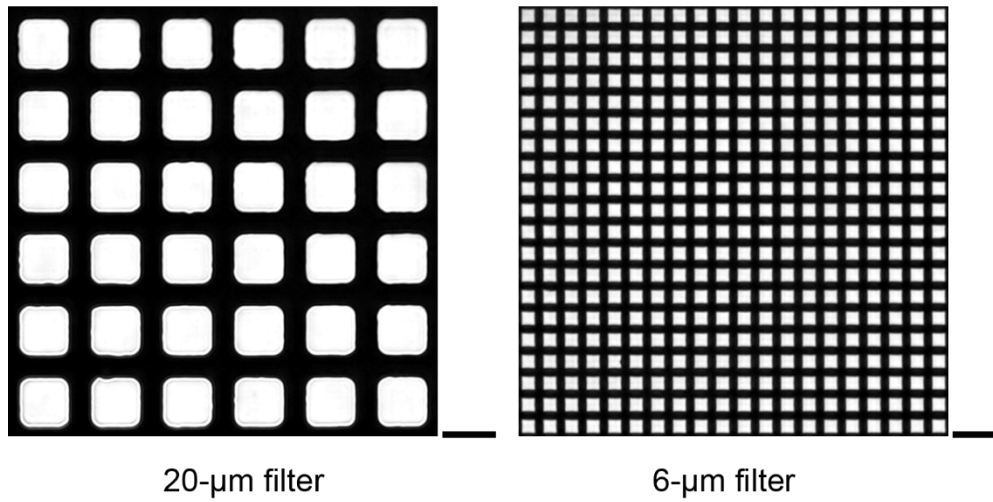
SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Fig. S1

Figure S1

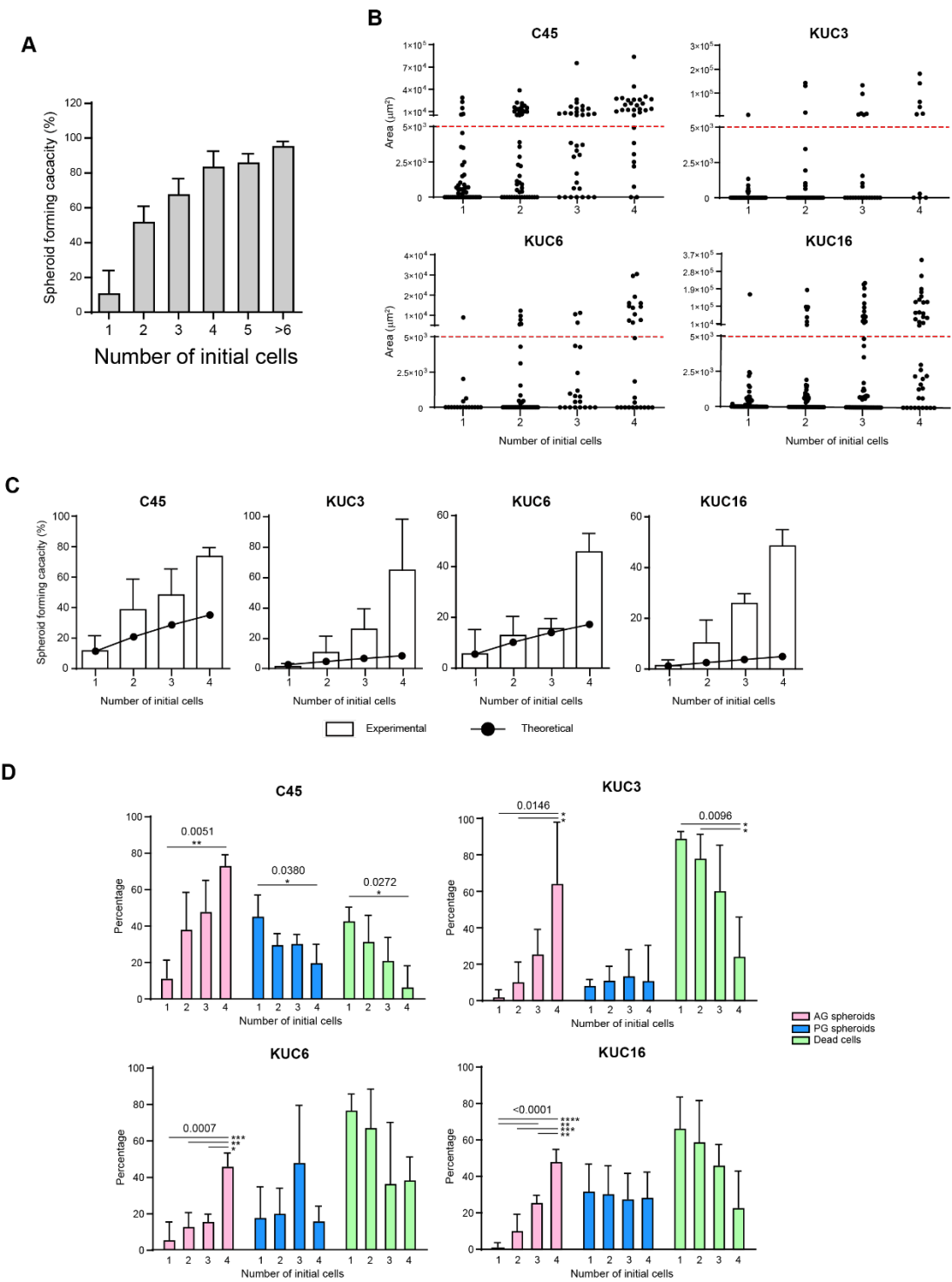


Microscopic images of filters for separating single cells and clusters

Microscopic images of 20-μm (left) and 6-μm (right) filters (CELLNETTA, Murata Manufacturing, Nagaokakyo, Japan) with high opening rates and accurate pore sizes. Scale bar: 20 μm.

Fig. S2

Figure S2

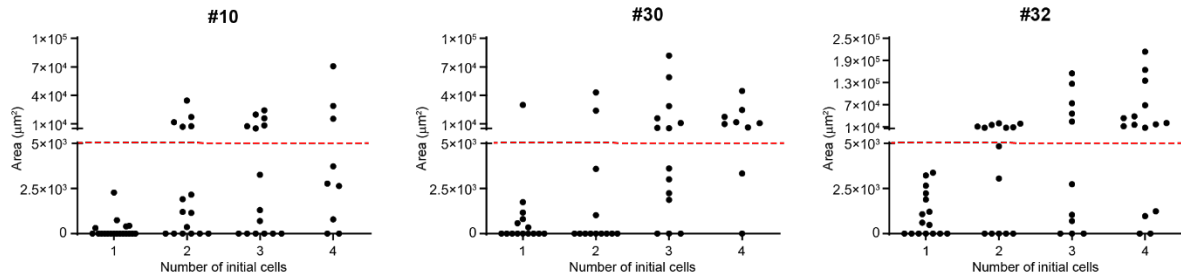


Growth patterns of cancer cells derived from CRC CTOS organoids as single cells and clusters

(A) Bar graphs of spheroid-forming capacity by numbers of cells at the beginning of the sphere-formation and growth (SFG) assay. Mean and SD is shown. Spheroid-forming capacity is the ratio of the number of wells containing actively growing (AG) spheroids to the total number of wells containing single cells or clusters at the start of the assay. (B) Scatter plot of the size of each SFG spheroid on day 14 (C45) and 21 (KUC3, 6, and 16) of the SFG assay. The threshold separating the AG and PG groups is indicated by the dashed red line. (C) Bar graphs of spheroid-forming capacity by numbers of cells at the beginning of the sphere-formation and growth (SFG) assay. Mean and SD is shown. Based on the assumption that only stem cells can form spheroids, the theoretical spheroid-forming capacity of the clusters was calculated. Theoretical spheroid-forming capacity was calculated by the following equation and shown as a line graph: $1-(1-\text{plating efficiency of single cells})^n$, n =initial number of cells. (D) Bar graphs of Fig. 1D. Mean and SD is shown. *P* values for ANOVA tests are indicated when statistically significant. Individual significance by Tukey's test is indicated by asterisks.

Fig. S3

Figure S3

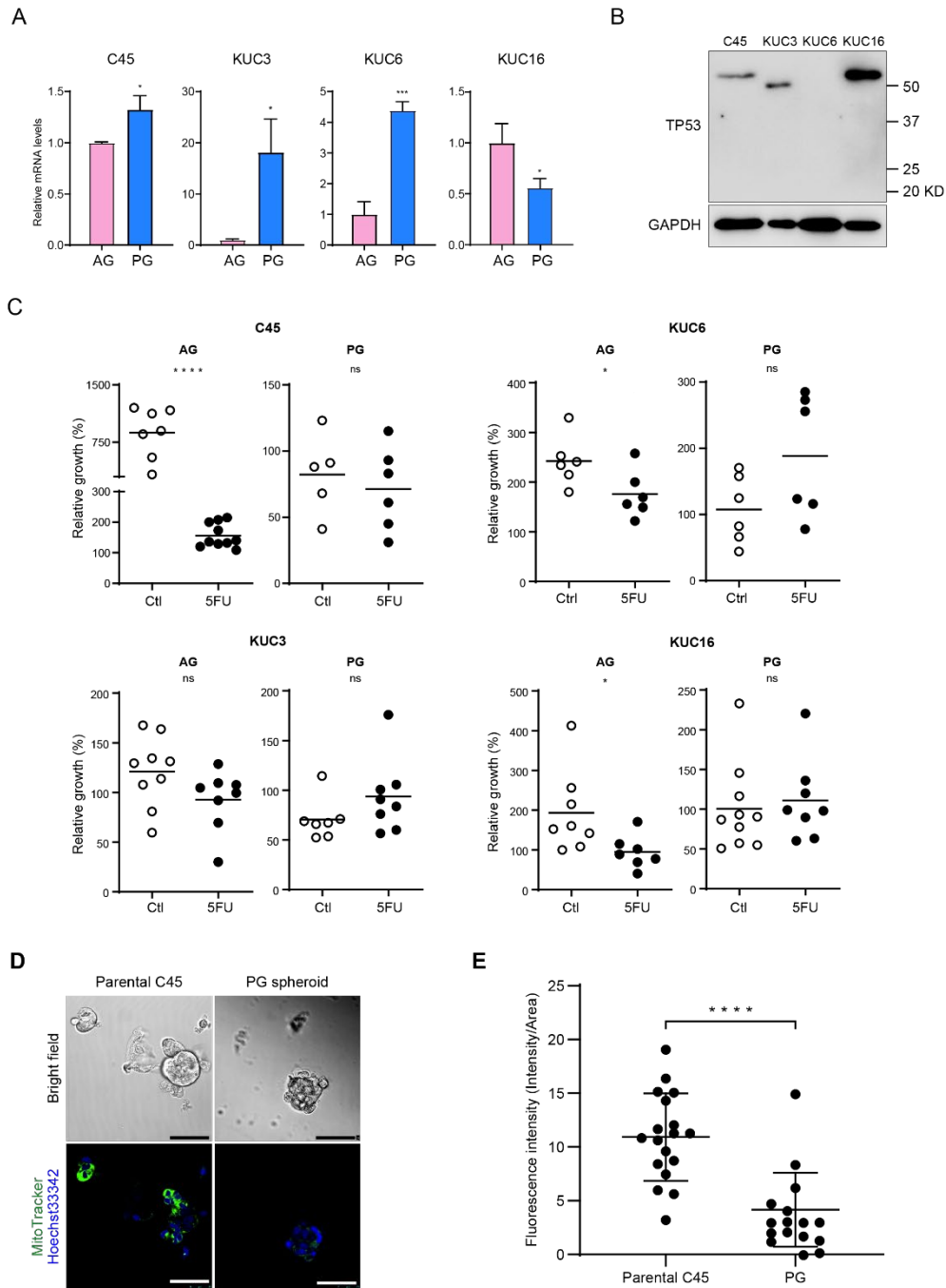


Growth pattern of the growing spheroids in PG group

Scatterplot of the size of each SFG spheroid in the SFG assay of the growing clones (Fig.2G). The threshold separating the actively growing (AG) and PG group is indicated by the red dashed line.

Fig. S4

Figure S4



Change of TP53 expression, chemosensitivity and mitochondrial activity in the SFG spheroids

(A) Semi-quantitative RT-PCR of *TP53* in actively growing (AG) and poorly growing (PG) spheroids on day 16 of the sphere-formation and growth (SFG) assay. *P* values for the Student's *t*-tests are indicated by asterisks. (B) Western blot of TP53 in the CTOS organoid lines. Molecular weights are shown on the left. (C) 5-FU sensitivity test. Scatterplot of the relative growth of AG and PG spheroids of four indicated lines after 7 days of 5-FU treatment. Dots represent the values for each SFG spheroid. The mean is indicated by a line. *P* values for Student's *t*-tests are indicated by asterisks. (D) Representative microscopic images of brightfield (top panels) and MitoTracker (bottom panels). Green, MitoTracker; blue, Hoechst33342. Scale bar: 50 μ m. (E) Quantification of (D). MitoTracker fluorescence intensity per area of the size-matched C45 and PG spheroids is shown. Each dot represents a spheroid.

Fig. S5

Figure S5

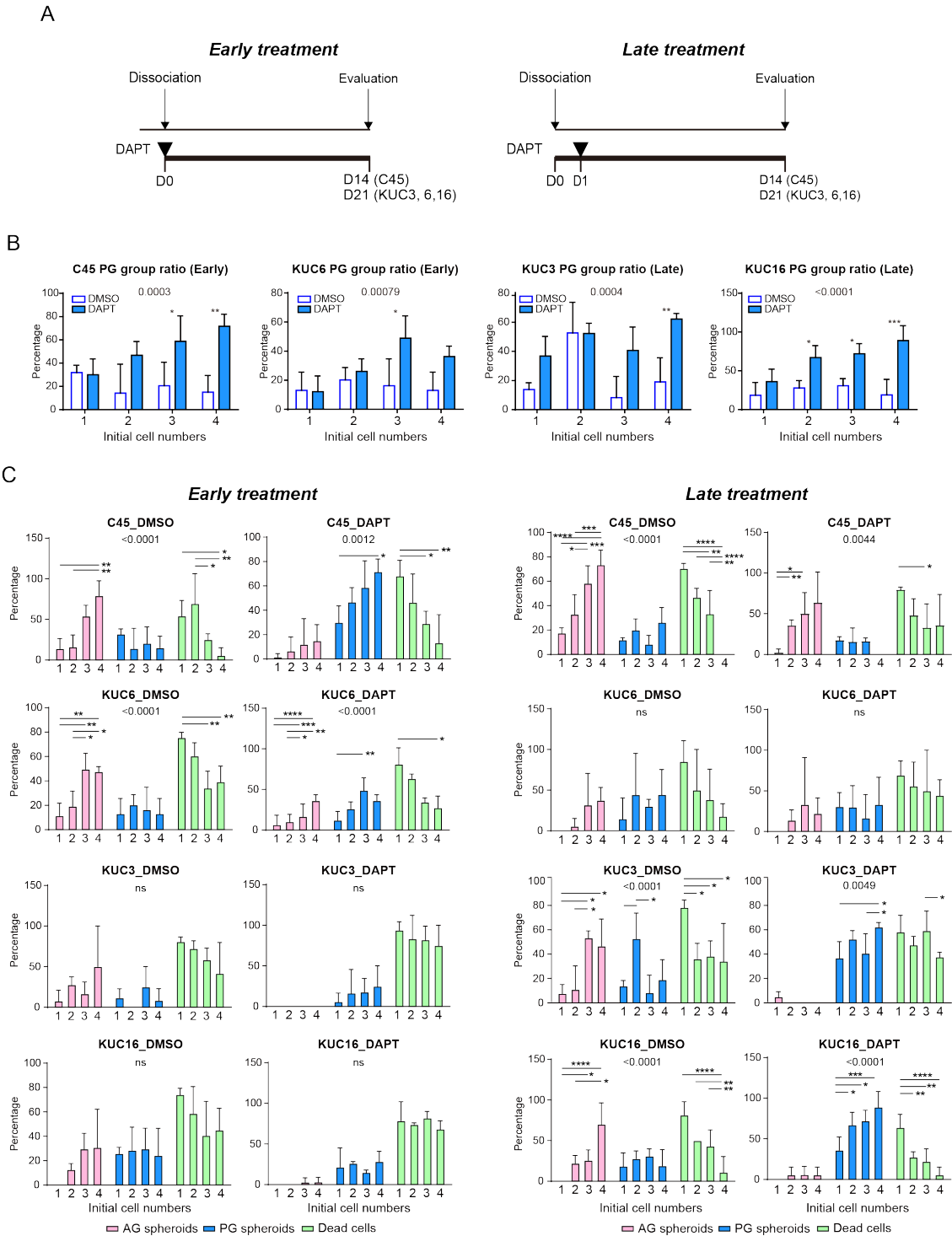
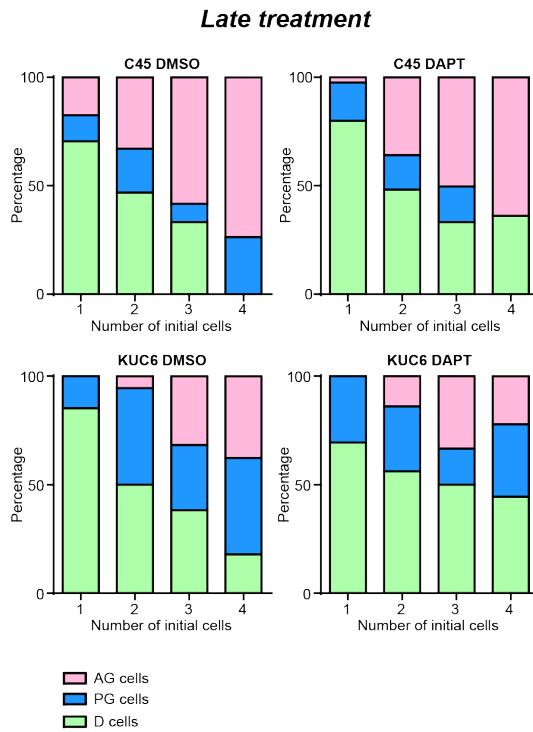
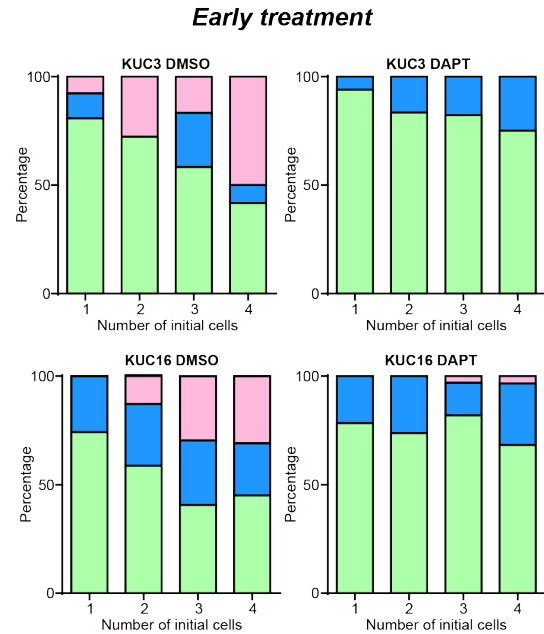


Figure S5

D



E

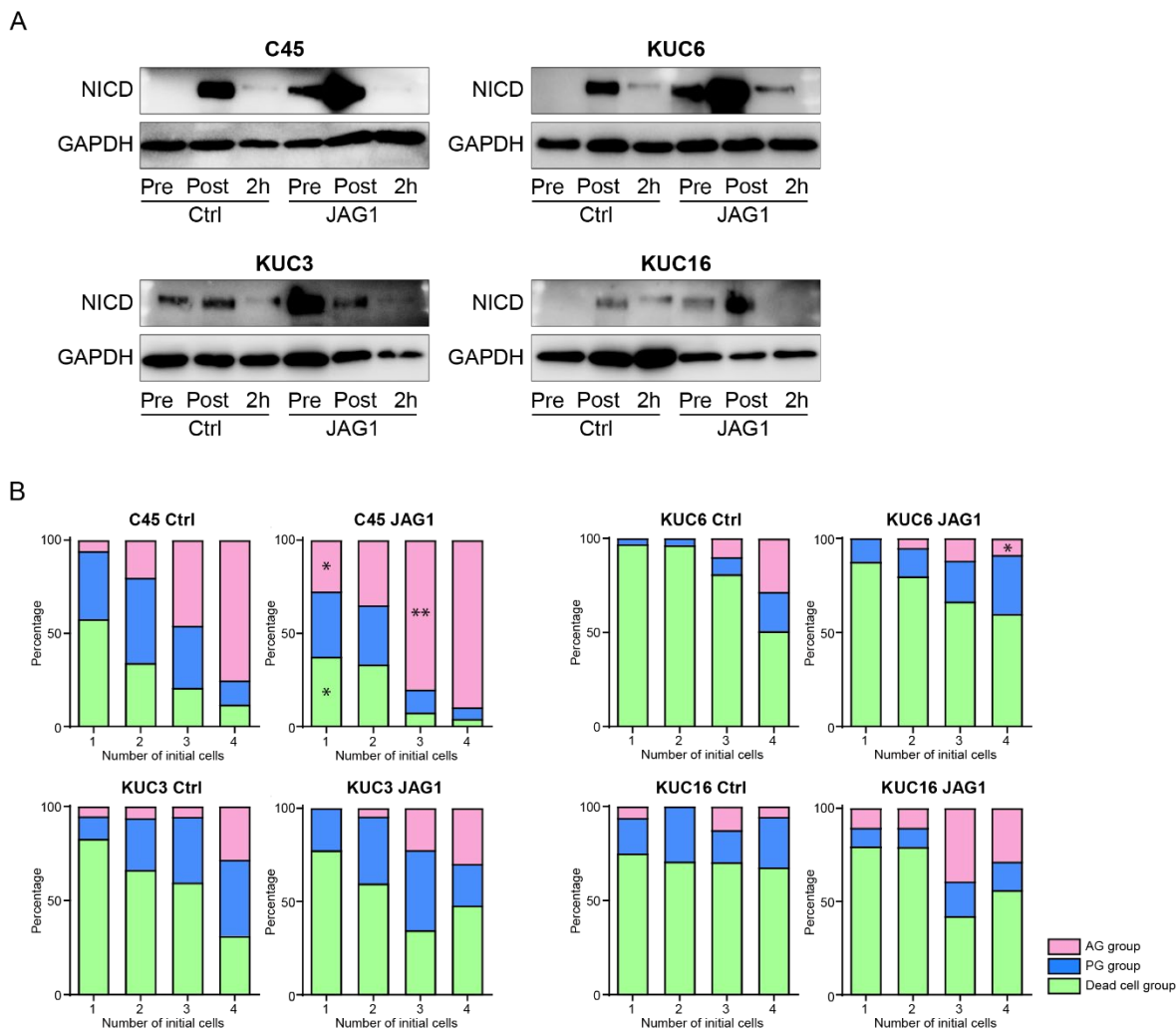


Effect of timing of Notch inhibition on sphere-formation and growth assay growth pattern

(A) Protocols of DAPT treatment. Early treatment started immediately after CTOS organoid dissociation and late treatment started 1 day after dissociation. (B) Comparison of ratios of poorly growing (PG) groups of (Fig.5C). *P* values for ANOVA tests are indicated when statistically significant. Individual significance by Tukey's test is indicated by asterisks. (C) Bar graphs showing the percentages of the three growth patterns in Figure 5C, S4D, and S4E. DAPT was added immediately after dissociation (early) or 1 day after CTOS organoid dissociation (late) as indicated. *P* values for ANOVA tests are indicated when statistically significant. Individual significance by Tukey's test is indicated by asterisks. (D, E) Stack graphs showing the ratio of growth patterns on day 14 (C45) or 21 (KUC3, 6, 16). DAPT was added 1 day after CTOS organoid dissociation (late) for C45 and KUC6 (D) and immediately after dissociation (early) for KUC3 and KUC16 (E).

Fig.S6

Figure S6

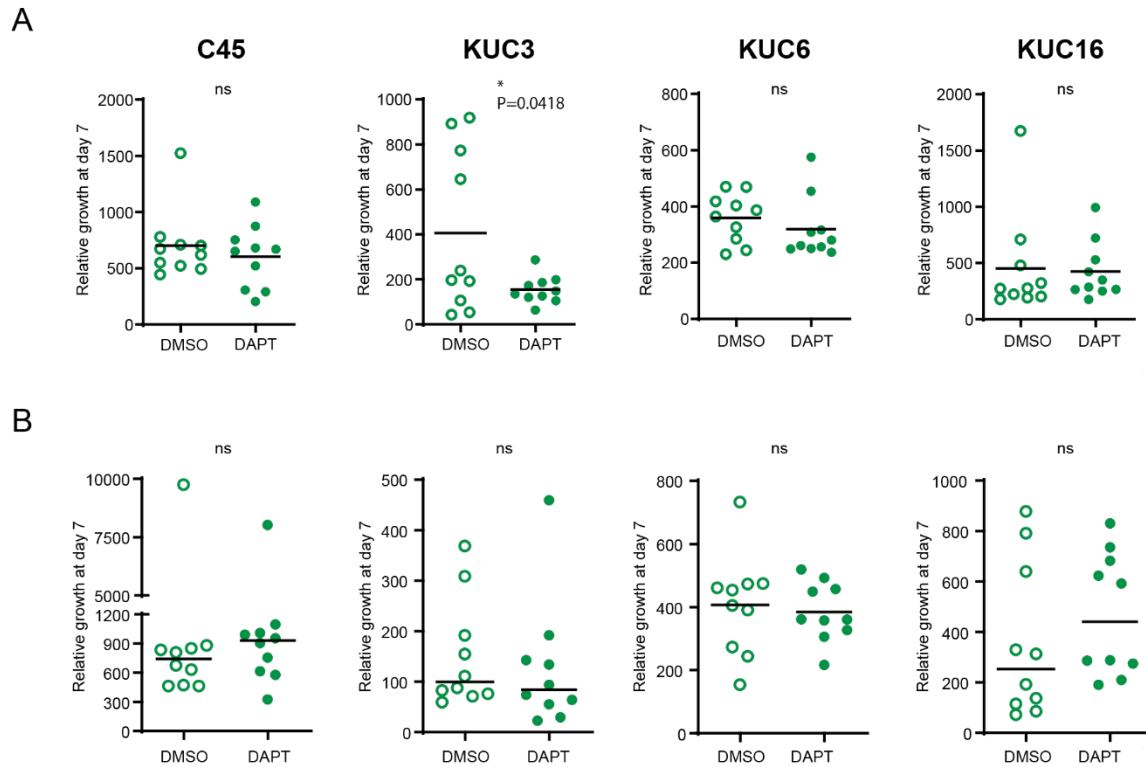


Effect of Notch activation by JAG1 on sphere-formation and growth assay growth pattern

(A) Western blot of NICD with and without JAG1 treatment. CTOS organoids 40-100 μm in diameter were pretreated with 1 μM recombinant human JAG1 (AnaSpec, Inc, AS-61298) for 2 hours and some were collected as Pre control (Pre). The remaining CTOS organoids were dissociated using the same protocol as the SFG assay. Some of the dissociated cells were collected as post, and the remaining cells were incubated for an additional 2 hours with 1 μM JAG1 (2 h). (B) Stack graphs of SFG assay with or without JAG1 showing the ratio of growth patterns on day 14 (C45) or 21 (KUC3, 6, 16).

Fig.S7

Figure S7



Direct effect of Notch inhibition on the growth of CTOS organoids

Scatterplots of relative growth (percentage of initial size) of each CTOS organoid 7 days after DAPT treatment. The initial diameter of each organoid ranged from 40 to 100μm (**A**) and 20 to 40μm (**B**).