Control of lumen geometry and topology by cell proliferation rate and pressure

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Abstract

Many internal organs in multicellular organisms comprise epithelia which enclose fluid-filled cavities referred to as lumens. Lumen formation is regulated by a wide range of processes, including epithelial polarization, secretion, exocytosis and actomyosin contractility. While these mechanisms have shed light on lumen growth, what controls lumen shape remains enigmatic. Here we used pancreas organoids to explore how lumen acquire a spherical shape or a branched topology. Combining computational simulations based on a phase field model and experimental measurements we reveal that the balance between the cell-cycle timing and lumen pressure are critical in regulating lumen morphology. Moreover, we show that by manipulating proliferation and lumen pressure *in-silico* and *in-vitro*, we can alter the morphological trajectories of the lumen during culture growth. Furthermore, we highlighted the crucial role of epithelial permeability, showing that increased permeability led to lower lumen pressure and more complex shapes. Artificially increasing permeability transformed spherical organoids into branching-like structures. In summary, the study underscores the importance of balancing cell proliferation, lumen pressure, and epithelial permeability in determining lumen morphology, providing insights relevant to other organs, for tissue engineering and cystic disease understanding.

Main

Internal organs frequently comprise epithelia that delineate fluid-filled lumens. These lumens vary in shape, ranging from sacs like the bladder to single tubes such as the intestine, or complex networks as seen in the kidney or in many glands including the pancreas [1]. These luminal and ductal structures play a pivotal role in organ functionality, serving as essential transport and delivery networks; any dysmorphogenesis in these structures can lead to severe pathological conditions [2].

Studies exploring lumen formation have leveraged various model systems, including zebrafish, Drosophila and mouse, to understand how these luminal and ductal structures form accross different developmental stages [3, 4]. Additionally, investigations in cell lines cultured in 3 dimensions (3D) such as the MDCK (Madin-Darby canine kidney) system have provided valuable insight into lumen morphogenesis [5]. In these systems, lumen morphology manifests as a single sphere. Organoid models offer the advantages of being more similar to physiological organs and while most of them have a single spherical lumen, several exhibit lumen with more complex geometries or topologies [4, 6–9]. These comprise diverticuli around a spherical core, single elongated tubes, multiple small lumen or networks of thin tubes [5, 10–12].

The intricate process of lumen morphogenesis involves multiple cellular mechanisms such as epithelial polarization, secretion, vesicle trafficking and fusion, cortical contractility, as well as cell death and rearrangement [7]. Numerous proteins controlling these processes have been identified. In addition, recent focus on the physics of lumen formation, notably the balance of luminal forces, such as hydrostatic pressure and cell mechanics has provided a more biophysical view of luminogenesis [8, 10, 11, 13–18]. These studies have largely been limited to spherical lumen in spite of recent insights regarding the formation of crypts, buds and multiple cavities.

We have previously showed that pancreatic mouse organoids can form either large spherical lumen or narrow complex interconnected lumen structures depending on the culture medium [12]. In this study, we investigate how morphological trajectories arise and can be altered.Combining experimental insights with predictive power of multicellular phase-field modeling, our assessment of proliferation and lumen pressure through targeted interventions unveils how the balance between cell-cycle timing and luminal pressure orchestrates the diverse spectrum of lumen morphologies.

Results

Two distinct morphological lumen trajectories in pancreas organoids.

Our previous work has shown that dissociated cells from embryonic day (e)10.5 pancreatic buds can generate spherical single-lumen and branching complex-lumen organoids in different media (hereafter, called spherical organoids and branching organoids for simplicity) (Supplementary Figure 1a) [12]. Specifically, the epithelium of the branching organoids forms a multilayered and branched structure by day 6, whereas the spherical organoids maintain their characteristic epithelium monolayer throughout culture growth (Figure 1a).

Apico-basal polarity plays an essential role in lumen formation and maintenance [7]. Disruptions in apicobasal polarity, whether genetic or chemical, often lead to anomalies in lumen characteristics, including the number of lumens [19, 20]. Monitoring the acquisition of apico-basal polarity in both organoid systems, we observed no significant differences at 24 hours and 48 hours (Supplementary Figure 1b). Divergence in their lumen morphology became evident from day 2 onwards, where spherical organoids typically developed and maintained a single lumen throughout the culture growth whereas branching organoids frequently formed multiple lumens (Figure1b & c). When multiple lumens were present, a larger star-shaped lumen and smaller peripheral lumens were observed. Our previous work has shown that lumens form in two ways in this system, either by cavitation between the small number of cells that seed an organoid or at the time of cell division at the abscission point (Supplementary Figure 1c) [9, 12]. On day 2, the number of lumen formed was proportional to the number of cells in a branching organoid (Figure 1d). Notably, the average number of lumens in the branching organoids peaked on day 2 before decreasing, with 3D reconstructions. This revealed the progressive formation of a hyperconnected network most likely due to the emergence of connections between lumens (Figure 1e).

To further elucidate lumen evolution, we analyzed lumen occupancy, which is defined as the 3D volume of the lumen relative to the total organoid volume (expressed as a percentage in Figure 1f) [9, 21]. Both organoid systems showed increasing lumen occupancy over time. Yet from day 2 onwards, branching organoids consistently exhibited lower lumen occupancy compared to spherical organoids. The branching organoids displayed higher number of cells throughout culture (Figure 1g and Supplementary Figure 1 d).

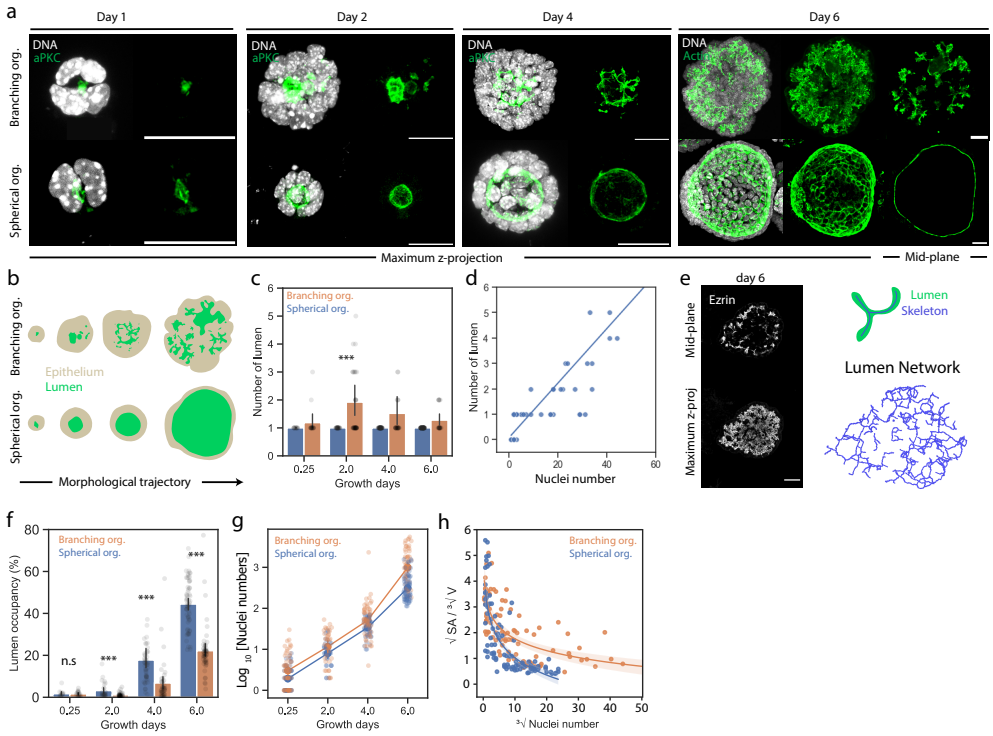

Figure 1. Divergent lumen shape and topology trajectories in pancreatic spheres and organoids (a) Max projection images of spherical and branching organoids at day 1, day 2, day 4, and day 6 of culture growth in Matrigel. Green: apical marker aPKC (day 1-4) and Actin (day 6), white: DNA. Scale bar = 20 μm. **(b)** Schematic representing the morphological trajectories of branching and spherical organoids. Beige: epithelium and green: lumen. **(c)** Number of lumen (quantified in 3D) of spherical and branching organoids at various days of culture growth. **(d)** Relationship between lumen number and cell number of branching organoids at day 2. N=3, n=92 branching organoids. **(e)** Left: Immunofluorescence images showing branching organoids at day 6 with Ezrin marking the lumen. Right: schematic showing the skeletonization of a lumen (top) to obtain a 3D lumen network (bottom) depicting lumen topology. Scale bar=20 μm. **(f)** Lumen occupancy in percentage (quantified in 3D) of spherical and branching organoids at various days culture growth of culture growth. N = 3-5 exp; n = 220 branching organoids, 194 spherical organoids. Error bar: 95% confidence intervals. **(g)** Quantification of number of nuclei (expressed as $\log_{10}$[number of nuclei] of branching and spherical organoids. N=3-5 exp; n=220 branching organoids, 194 spherical organoids. **(h)** Quantification of the relationship between the lumen surface : volume ratio ($\sqrt{surface\,area}$: $\sqrt[3]{volume}$ per lumen) vs number of nuclei ($\sqrt[3]{nuclei\,number}$ per organoid). Linear regression estimated of the form lumen surface:volume ratio $\sim$ log(number of nuclei). N = 3-5 exp, n = 165 branching organoids, 194 spherical organoids. The Mann-Whitney test was performed for all statistical significance tests.

As cells constitute the lumen surface area, a higher cell number and lower lumen occupancy result in an increased surface-to-volume ratio in branching organoids (Figure 1h) . In contrast, spherical organoids minimize their surface-to-volume ratio, maintaining their spherical lumen shape.

Phase field multicellular modelling and experimental validation reveal a major role of lumen pressure and cell cycle duration on the morphological diversity of lumens.

From the experiments above, branching and spherical organoids exhibit differences in both topological (number of lumens) and geometric features, with branching organoids showing larger surface-to-volume ratios. Since the increase in cell number was faster in branching organoids, we hypothesized that the creation of new lumen at cell division might drive differences in lumen shape and topology, notably leading to an increase in the number of lumens and apical surface. Additionally, considering studies indicating that lumen growth can be regulated by its pressure, we investigated whether the balance

between lumen pressure and epithelium proliferation constitutes a primary difference between these systems [13, 17, 21, 22].

To connect single cell dynamics (cell division and neighbor exchange) with luminal nucleation and growth, we turned to theoretical investigation by applying phase-field modelling. To mimic organoids, the model incorporates force balance for each cell, more specifically cortical surface tension, adhesion with neighboring cells, and pressure ξ by the neighboring lumen. As we observed differential growth in branching and spherical organoids, we included the parameter τ_V, the natural interval between cell divisions in the absence of mechanical constraints. Moreover, cell division orientation was determined by the force balance in the dividing cell (see methods for more details and [21].

The phase diagram given by the numerical simulations based on this model revealed that both cell cycle duration and pressure affected lumen shapes and numbers (Figure 2a & b). Multiple areas in the phase diagram displayed lumen features that were comparable to our branching and spherical organoids (Figure 2c). Branching organoids were found in regions corresponding to faster proliferation and lower pressure than spheres.

To experimentally test these observations and validate the model, we sought to characterize the difference in osmotic pressure and cell-cycle timing of both organoid systems. To address whether the branching organoids proliferate faster, we performed an EdU (5-ethynyl 2´-deoxyuridine) incorporation assay, labelling cells in S-phase within the organoids. We treated the organoids with EdU for 2 hours prior to fixation at 6, 24, 48, and 96 hours of culture growth (Figure 2d). To analyze the difference in proliferation, we quantified the ratio of cells that were EdU positive (S-phase) and the total number of nuclei. We observed that at 6 hours and 24 hours both branching and spherical organoids displayed comparable EdU:DNA ratios. However after 24 hours the spherical organoids exhibited a consistently lower relative number of EdU positive cells compared to the branching organoids (Figure 2e). As the parameter used in the model τ_V is the interval between cell divisions, we quantified the cell number in organoids and found that the branching organoids exhibited consistently higher average cell numbers over the duration of the culture period (Figure 2e). These experiments showed that both organoid systems increase their potential to proliferate and start diverging after day 1. This observation coincides with the similar lumen morphologies the two organoids share at day 2 (Figure1a & b). Since the interval between cell divisions τ_V was found to be an important control parameter in the numerical simulations, we calculated the doubling time for both organoid systems by utilizing the average cell number in organoids at later culture days, *i.e.*, when the average morphologies of the branching and spherical organoids where the most different (from day 4 onwards). (Figure 1a & b). Assuming exponential growth and neglecting contribution from cell death, we found that the doubling time of the branching organoids was 1.4 faster than that of the spherical organoids from 48 to 96 hours (Figure 2g).

Another control parameter which played a key role in the phase diagram was ξ (see Section 0.12 of Materials and Method). We, therefore, asked whether the branching and spherical organoids exhibited different lumen hydrostatic pressure. We performed laser ablations to infer hydrostatic pressure using the Hagen-Poiseuille equation [8, 17, 23, 24]. Laser ablation creates a conduit across the epithelium layer between the lumen and the external environment (Figure2i). We observed a decrease in the lumen volume measured in 3D-segmented lumen pre- and post-ablation indicating a higher hydrostatic pressure in the lumen than around the organoids (Supplementary Figure 3b). The fluid expelled from the lumen could be seen by including a dye in the medium surrounding organoids (Supplementary Figure 3a: right). To calculate the hydrostatic pressure difference, we quantified the flow rate based on the measurements of volume change, ablated conduit radius and height of the epithelium layer (Figure 2h) [8]. Since the calculation requires information on lumen fluid viscosity, we exploited the observation that when organoids were treated with Cell-Mask, Cell-Mask positive particles were observed in the lumen after 2 hours (Supplementary Figure 2a). These particles were segmented, and their movements tracked over time in 3D to acquire the mean squared displacement. Using these curves, we derived the diffusion coefficient of the lumen particles which were used to estimate the lumen's viscosity using the Stokes-Einstein equation (Supplementary Figure 2 b-g). We observed that the mean lumen fluid viscosity was 2.06±0.35 mPa·s. Measurements in 1% glycerol and water using this method revealed values of 1.39±0.22 mPa·s and 0.73±0.07 mPa·s, respectively, which are expected values (Supplementary

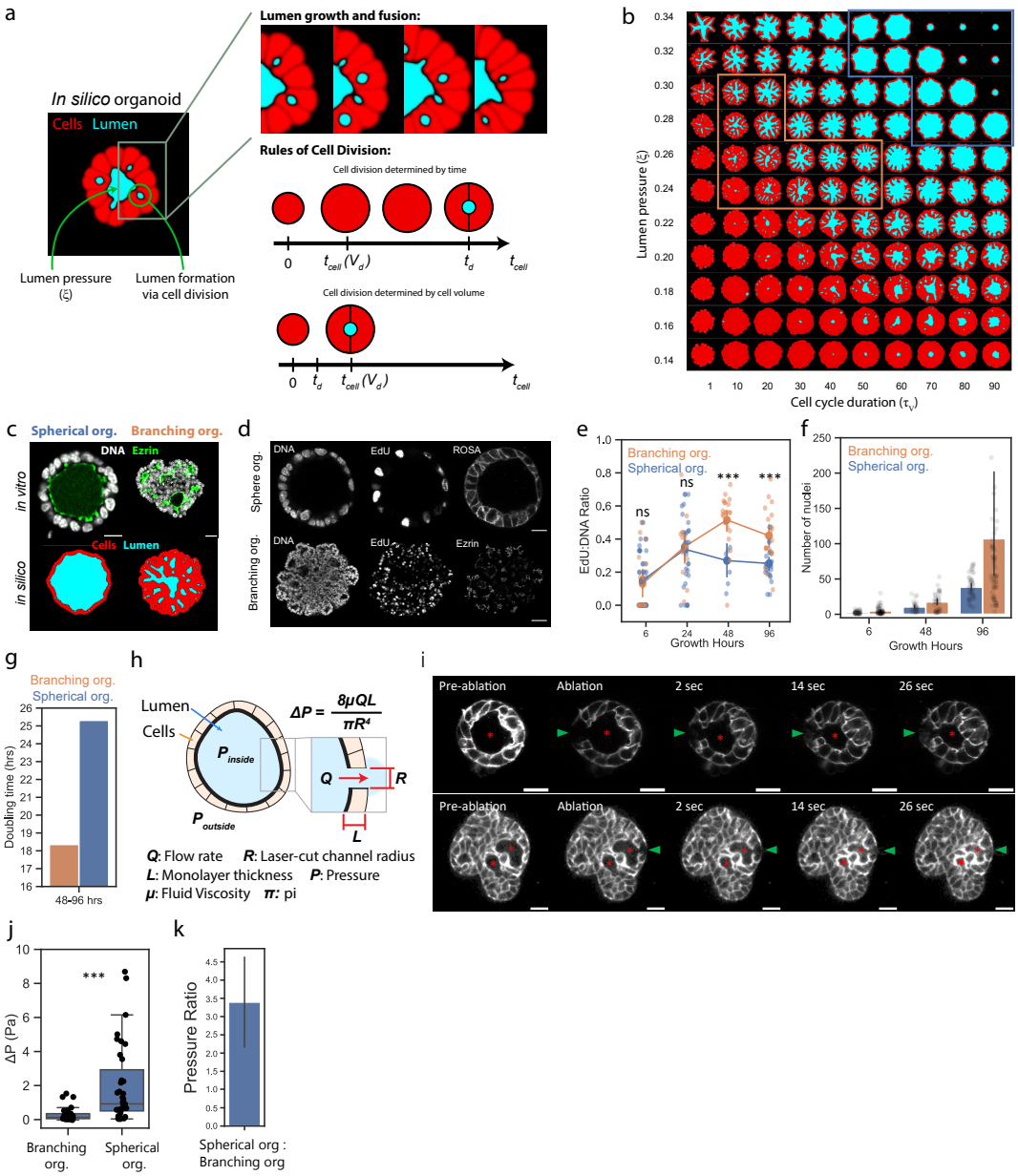

Figure 2. Organoids with complex lumen form at low pressure and fast proliferation rates. (a) Schematics describing the model and processes involved: lumen growth and formation via cell division. **(b)** Phase diagram. **(c)** Qualitative and morphological resemblance between *in-vitro* spherical and branching organoids (top row) and *in-silico* organoids. Scale bar = 20 µm. **(d)** Immunofluorescence images of spherical and branching organoids after 2 hours of EdU labelling. Scale bar= 10 µm (top row) and 30 µm (bottom row). **(e)** EdU:DNA ratio of spherical and branching organoids at various hours of culture growth. N = 2-5 exp; n = 72 branching organoids, 109 spherical organoids. Error bar: 95% confidence intervals. **(f)** Nuclei counts per organoid at various hours of culture growth. Error bar: 95% confidence intervals. N = 3 exp; n = 180 branching organoids, 129 spherical organoids. **(g)** Quantification of doubling time for branching and spherical organoids from 48 hours to 96 hours of culture growth. **(h)** Schematic of the laser-ablated organoid and measurements used in the Hagen-Poiseuille equation for lumen hydrostatic pressure inference. **(i)** Laser ablation experiment montage on a branching and spherical organoid expressing ROSAmTmG. Red asterisk: targeted lumen (connected lumen in 3D for branching organoid), green arrowhead: channel created by laser ablation. Scale bar = 20 µm. **(j)** Inferences of lumen hydrostatic pressure ΔP of the branching and spherical organoid lumens on day 6 of culture. N = 3 exp; n = 36 branching organoids, 34 spherical organoids. **(k)** Quantification of spherical organoid: branching organoids lumen hydrostatic pressure ΔP ratio. Error bar: standard error of the mean over average ratios (See Supplementary Figure 3f). The Mann-Whitney test was performed for all statistical significance tests.

Figure 2h - j). As the lumens in organoids were too narrow to make such measurements we assumed that the lumen viscosity in both organoid systems was similar and applied the average lumen viscosity obtained from the spherical organoids to infer ΔP of both systems. With the lumen viscosity, flow rate, and conduit dimensions created by the laser cut, we obtained mean ΔP of 0.28 Pa for the branching organoids and 1.46 Pa spherical organoids on day 6 of culture (Figure 2j).

Moreover, we observed that the ΔP of the lumen spherical organoids had a positive correlation with increasing volume of the lumen, which was not the case for the branching organoids (Supplementary Figure 3d). We thus compared lumen of the same size for organoids and spheres after binning lumen volumes (Supplementary Figure 3e & f). This method revealed that the ΔP of the lumen pressure in the size-matched spherical organoids is approximately 3.3 times higher than in branching organoids (Figure 2k).

Taken together these results show that organoids exhibit faster proliferation and lower pressure, as expected from the phase diagram regions with more branched lumen.

Controlling organoid lumen morphology through manipulation of lumen pressure and proliferation.

Given that our quantitative comparisons for proliferation and pressure involved organoids grown in different culture media, we aimed to specifically perturb proliferation and lumen pressure in the same organoid medium. To test the model predictions, we used aphidicolin which slows down or stops proliferation by inhibiting DNA Polymerase-A thus arresting cells in the S-phase of the cell cycle. Lumen morphogenesis is a slow process (time scales of days). Accordingly, short-term (10 hours) aphidicolin treatment was observed to have no impact on intestinal organoid morphologies [11]. Thus we treated the most proliferative of our organoid types, the branching organoids, with DMSO and aphidicolin at day 4 and analyzed them at day 6 (Figure 3a).

Under these treatment conditions, the cell cycle was totally arrested and we observed no or few organoids with pH3s10 positive cells (Supplementary Figure 4a and 4b). We were unable to identify a dose of Aphidicolin that slowed proliferation. By day 6, branching organoids exhibited a more spherical shape, fewer lumen and higher lumen occupancy compared to DMSO-treated branching organoids (Figure 3b & c). These results indicate that stopping proliferation has a drastic impact on lumen morphology. Since in the *in silico* experiments, increasing in τ_V (moving towards the right of the τ_V axis in the phase diagram in Figure 2b) involved reducing rather than stopping proliferation, we performed new simulations where cell cycles were stopped after organoids reached a cell number similar to the number we quantified in 2D at day 4, prior to aphidicolin treatment (Supplementary Figure 4c-i). Moreover, we compared experimental organoids with numerical model phenotypes . Under these conditions, the *in silico* organoids evolved into spheres with single large spherical lumens mirroring the lumen occupancy and numbers (Supplementary Figure 4e - g) observed in experiments (Supplementary Figure 4h-i).

Numerous studies underscore lumen pressure's influential role in determining organoid morphologies. Notably, cycles of inflation and collapse enable the formation of crypts in intestinal organoids [8, 17]. However, whether the lumen pressure or its volume evolution can directly impact lumen morphology has not yet been studied.

To test whether lumen pressure directly influences morphology, we treated the branching organoids with forskolin, an activator of cystic fibrosis transmembrane regulator (CFTR) ion channels which triggers the secretion of chloride ions and bicarbonate into the lumen. Forskolin treatment from day 4 to day 6 in our branching organoids resulted in inflated lumen and increased lumen occupancy but did not yield a single spherical lumen (Figure 3c - i). Although there was a 1.3 fold increase in hydrostatic lumen pressure was not significant (Figure 3g & Supplementary Figure 5a - d).

Epithelial permeability contributes to the regulation of lumen morphology.

Given the unexpected lack of lumen pressure increase by Forskolin, we hypothesized that the epithelium of organoids may not retain solutes and water sufficiently to enable pressure increase. Epithelial paracellular permeability or "tightness" regulates the epithelial barrier function against ions, solutes, molecules [25].

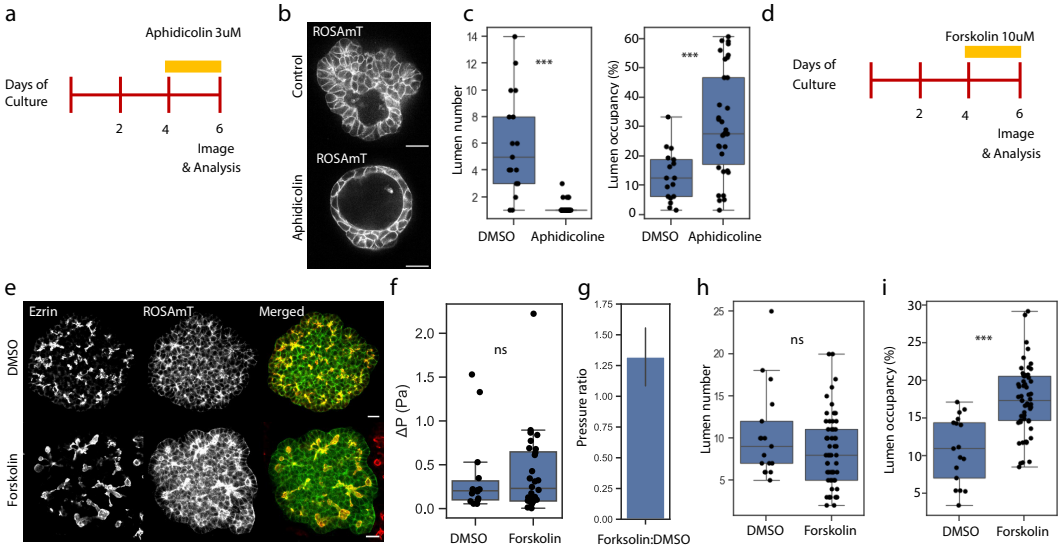

Figure 3. Interferences with cell cycle and ion secretion change lumen morphology and/or topology (a) Schematic showing experimental design of aphidicolin assay. **(b)** Immunofluorescence image of control and aphidicolin treated branching organoids at day 6. Scale bar = 20 µm. **(c)** 2D Quantification of lumen number (left) and lumen occupancy (right; in percentage) of DMSO and aphidicolin-treated branching organoids at day 6. N = 3 exp, n = 17 DMSO, 34 Aphidicolin. **(d)** Schematic showing experimental design of forskolin treatment. **(e)** Immunofluorescence image of DMSO and forskolin treated branching organoids at day 6. Scale bar = 20 µm. **(f)** Inference of hydrostatic pressure ΔP of the DMSO and Forskolin treated branching organoid lumens on day 6 of culture. N = 3 exp; n = 26 DMSO, 29 Forskolin. **(g)** Hydrostatic pressure ratios of forskolin: DMSO treated branching organoids. Error bar: standard error of the mean. **(h)** 2D Quantification of lumen number of DMSO and Forskolin treated branching organoids at day 6. N = 3 exp, n = 27 DMSO, 31 Forskolin. **(i)** 2D Quantification of lumen occupancy (right; in percentage) of DMSO and Forskolin treated branching organoids at day 6. N = 3 exp, n = 27 DMSO, 31 Forskolin. The Mann-Whitney test was performed for all statistical significance tests.

To test this hypothesis, we performed a permeability assay by adding 10kDa fluorescent Dextran-647 into the medium. Comparable to reports of MDCK and certain intestinal organoids, the spherical organoids displayed no or low signals of 10kDa Dextran-647 in the lumen after 3 hours, indicating their "tight" epithelial monolayer [8] . However, branching organoids displayed lumens with 10kDa Dextran-647 signals as well as signal in paracellular spaces indicating higher permeability (Figure 4a)..

To investigate whether increased permeability leads to decreased pressure and a subsequent change in lumen formation, we used Clostridium perfringens enterotoxin (CPE) which forms pores in the epithelium by binding to claudins [25,26]. Non-cytotoxic recombinant forms of CPE (cCPE) manipulate barrier functions via tight junction modulations (Figure 4b).

Reports show that CPE has the highest affinity towards Claudin-3 and -4, and both organoid systems express prominent levels of the two claudins based on transcriptomics (Supplementary Figure 6a). Therefore, we synthesized cCPE labelled with ATTO647 maleimide and used it to manipulate permeability and investigate alterations in lumen morphology (Figure 4b). Within 2 hours of cCPE-647 treatment, signals were detected in the paracellular spaces of spherical organoids followed by cCPE-647 puncta in the cytoplasm, consistent with previous reports of internalization with claudin targets [27] (Figure 4b & c). We then co-treated spherical organoids with 3-5 kDa Dextran-488 and cCPE-647 to assess permeability and monitor alterations in the lumen. Approximately 1 hour after cCPE-647 treatment, we observed an increase in the level of 3-5 kDa Dextran-488 in the lumen, coinciding with an increase in the lumen area (Figure 4d & e). After approximately 2 hours post-treatment, when the 3-5 kDa Dextran-488 levels in the lumen plateaued, we observed a shrinkage in the lumen area. Using laser-cutting, we observed that cCPE-647 lowered the lumen hydrostatic pressure (mean 1.15Pa) compared to the control condition (mean 2.22Pa) (Figure 4h).

To evaluate the long-term effects of induced-permeabilization and reduced pressure on lumen morphogenesis, we treated spherical organoids in sphere medium with cCPE-647 from day 2 to day 6 (Figure 4f) and observed 50% of organoids adopting a branching morphology, exhibiting lower lumen occupancy and higher lumen number (Figure4g - k & Supplementary Figure 6b - c). No change in cell number was observed. Moreover, overall shape of the organoid shell was more branched, as shown by a decreased sphericity (Supplementary Figure 6f - g).

Collectively, these experiments indicate that permeability has a significant role in regulating lumen pressure with consequences on lumen morphology, independently of cellular proliferation.

Discussion

While previous work has highlighted the importance of apical growth via vesicle fusion is crucial to in the formation and enlargement of lumen in the pancreas and other 3D lumen models, our study unveils another critical factor shaping pancreatic: the permeability of the pancreatic epithelium and its role in establishing and maintaining a moderate lumen pressure [28–32]. A balance between high junctional tension and low lumen pressure has been reported to affect lumen shape in ZO-1/2 knockout MDCK cysts, creating invaginations in the apical membrane of cells lining the lumen. In contrast Claudin 1-5 knockouts exhibited no such change in lumen morphology and no change in pressure [8,16]. Importantly, our experiments with cCPE, a claudin-targeting toxin, further substantiate that altering lumen permeability can significantly change hydrostatic pressure and consequently lumen morphology. These findings suggest that different claudins may mediate distinct permeability characteristics, as observed in various intestinal organoid models with different tight junction compositions showing differential permeability to Dextran [8,16,33]. Tissue specificities in the balance of mechanisms controlling pressure, apical growth and junctional tension would require further investigations in different organs and organoid proxys. In this study, focusing on the pancreas, the change in lumen shape at different pressure regimes is unique, and rewires between extreme trajectories such as single spherical lumen and thin interconnected luminal networks upon permeability and pressure manipulations. It is likely that a balance between permeability to specific solutes and the rate at which they are secreted in the lumen controls the luminal pressure and thereby shapes the lumen.

Another significant parameter influencing lumen shape is the cell division rate, which dictates the

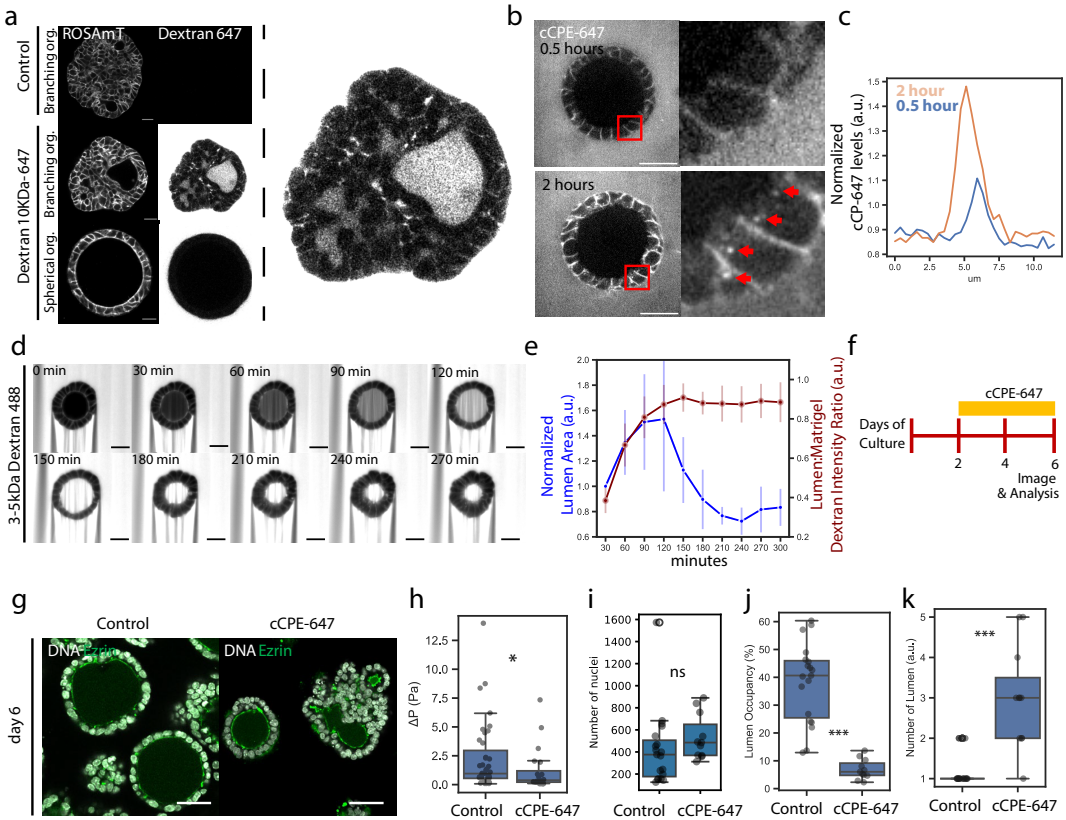

Figure 4. Transformation of spherical organoids into branching organoids through induced permeation. (a) Left: Live images of control and 10kDa Dextran-647 treated live branching and spherical organoids 3 hours post-treatment. Right: Enlarged image of branching organoids with 10kDa Dextran-647 signal detected in paracellular spaces and lumens. Scale bars = 20 µm. **(b)** Live images of day 4 spherical organoids treated with cCPE-647 at 0.5 hours and 2 hours of incubation. Fluorescent signals are cCPE-647 in the Matrigel and paracellular spaces. Red arrowheads indicate cCPE-647 puncta in the cytoplasm. Scale bar = 20 µm. **(c)** Line-profile quantification of cCPE-647 (normalized to signals in the Matrigel) across the paracellular spaces at various time points of treatment. **(d)** Montage of live spherical organoids co-treated with Dextran 3-5 kDa-488 and cCPE-647 at various timepoints of treatment. Scale bar = 20 µm. **(e)** Quantification of normalized levels 3-5kDa Dextran-488 in the lumen (mean lumen: mean Matrigel intensity ratios of Dextran 3-5 kDa-488) and mean normalized 2D area of lumen (normalized to lumen area at first time point of image acquisition) at various time points of cCPE-647 treatment. n = 5 spherical organoids. Error bar: standard deviation. **(f)** Schematic showing experimental design of cCPE-647 treatment. **(g)** Immunofluorescence images control and cCPE-647-treated spherical organoids on day 6. Scale bar = 30 µm. **(h)** Inference of hydrostatic pressure ΔP of the control and cCPE-647 treated spherical organoid lumens on day 6 of culture. N = 2-3 exp; n = 39 control, 20 cCPE-647. The Mann-Whitney test was performed for statistical significance. **(i-k)** 3D quantification of nuclei counts, lumen occupancy, and number of lumens of control and cCPE-647 treated spherical organoids on day 6. N = 2-3 exp; n = 27 control, 17 cCPE-647. The Mann-Whitney test was performed for all statistical significance test.

rate at which new lumen form at abscission points. Cerruti *et al.*, have used cell packing patterns and modelling to evaluate how far from mechanical equilibrium MDCK cysts are. They further showed that this depends on their cell division rate and their cell rearrangement rate, with the latter being at a longer timescale than duplication [19]. While their interference experiments showed the importance of cell rearrangement rate, their inhibition of cell division did not reveal drastic changes in lumen shape or topology. This difference can be attributed to the higher pressure regime in MDCK cysts, promoting more spherical morphologies, potentially masking the effects of cell proliferation [8]. In contrast, our study shows that in pancreatic organoids, cell division rate significantly impacts lumen shape. We hypothesize that in branching organoids, lumen volume cannot expand quickly enough to match the increasing apical surface area provided by rapid cell cycles, resulting in multiple small lumens rather than one large lumen (Supplementary Figure 1d). Additionally, we observed that the cell rearrangement rate is too slow to promote the formation of a single lumen from the coarsening of multiple lumen.

Collectively, this study highlights the critical influence of the culture medium composition on modulating physical parameters regulating the morphogenic trajectories of pancreatic organoids. The high levels of FGFs and EGFs in the organoid medium promote fast cell division. We propose that rapid lumen creation is only partially compensated by slow cell rearrangements, potentially contributing to the leakiness of the system, as cells must continually reorganize their junctions. In the slow division regime we propose that the cells rearrange less and that the pressure increases due to apical ion secretion in the lumen. In spherical organoids, cells tend to divide within the plane of the epithelium because of the large luminal pressure, directly contributing new apical material to the main lumen. This suggests that luminal pressure may influence the division angle, creating a feedback mechanism that maintains a spherical lumen shape.

Our study employs a reductionist 3D culture model using primary cells freshly isolated from *in vivo* tissues, enabling experiments that would be challenging to perform *in vivo*. Despite its simplicity, this model system reveals that a finely-tuned balance between cell proliferation, lumen pressure, and epithelial permeability dictates the morphological diversity observed in pancreatic organoids. It reveals mechanisms that are potentially relevant to other organs exhibiting narrow ducts and to common cystic diseases affecting various branched organs, paving the way for future research and possible therapeutic interventions targeting these processes in tissue engineering and regenerative medicine.

Materials and Methods

0.1 Animals and permit

All experiments were performed in accordance with the German Animal Welfare Legislation ("Tierschutzgesetz") after approval by the federal state authority Landesdirektion Sachsen (license DD24.1-5131/451/8). Mice were kept in standardized specific-pathogen-free (SPF) conditions at the Biomedical Services Facility (BMS) of Max Planck Institute of Molecular Cell Biology and Genetics. Genetically modified mouse lines LifeAct-EGFP [34] and 'Gt(ROSA)-26Sortm4-(ACTB-tdTomato-EGFP)-Luo(C57Bl/6NCrl;Crl)'.

0.2 Pancreatic organoid culture

Mouse embryonic stage e10.5 was defined as noon of the day when the vaginal plug was detected in the mother. Pancreatic buds were dissected from e10.5 mice and mesenchymal cells were removed using Tungsten needles [35]. To obtain cell aggregates, the buds were dissociated using TrypLE (12604013, ThermoFisher Scientific) treatment for 12 min in a 37°C incubator, followed by mechanical dissociation using pulled glass capillaries (BR708707, BRAND/Merck). The cell aggregates were seeded into 75% Matrigel (356231, Corning) in 8 well glass bottom plates (80826, Ibidi) and left to polymerize at 37°C in an incubator for 10 min. To grow branching organoids, a medium composed of 25 ng/mL murine-FGF1 (450-33, Perprotech), 25 ng/mL murine-EGF (315-09, Perprotech), 2.5 U/mL Heparin (7980, Stemcell), 10 μM Y-27632 -dihydrochloride ROCK inhibitor (Y0503, Sigma Aldrich), 16 nM

Phorbol-12-myristat-13-acetate (524400, Milipore), 100 ng/mL murine-FGF10(450-61, Perprotech), 500 ng/mL murine-Spondin-1 (315-32, Perprotech), 10% Knockout serum (10828-028, Gibco), 1% Penicillin-Streptomycin (15140-122, Sigma Aldrich) and DMEM/F12 (1:1) 1x (+) L-Glutamine (11320-033, Gibco) was added. To grow spherical organoids, a medium composed of 64 ng/mL murine-FGF2 (450-33, Peprotech), 10% B27 supplement (17504-044, Gibco), 10 μM Y-27632 -dihydrochloride ROCK inhibitor (Y0503, Sigma Aldrich), 1% Penicillin-Streptomycin (15140-122, Sigma Aldrich), and DMEM / F12 (1:1) 1x (+) L-Glutamine (11320-033, Gibco) was added. The organoids were grown for 6 days in culture in an incubator at 37°C 5% CO_2. Medium exchange was carried out every 2 days.

0.3 Pharmacological & Chemical treatment

To stop proliferation, pancreatic organoids were treated with aphidicolin (32774, Cell Signaling Technology/Merck), a DNA Polymerase A inhibitor. Aphidicolin was dissolved in DMSO (800153, Cell Signaling Technology) and a final concentration of 3 µM concentration were used for 2 days. To inflate the lumen, the CFTR activator forskolin (32774, Tocris Bioscience) was used. A stock concentration of 20 mM was achieved by dissolving forskolin in DMSO. For pancreatic organoid treatment Forskolin was diluted to 10 µM for 2 day-long treatments.

0.4 Immunofluorescence

Pancreatic organoids were fixed with 4% Formaldehyde (28908, ThermoFisher Scientific) in PBS for 30 min at room temperature. Samples were blocked and permeabilized in 0.25% Triton (T8787, Sigma Aldrich), 1% bovine serum albumin (BSA; A3059, Sigma Aldrich) in PBS for 6 h at room temperature. Pancreatic organoids were incubated in primary antibody solution in 0.25% Triton, 1% BSA in PBS overnight at 4°C, and in secondary antibody solution in 0.25% Triton, 1% BSA in PBS overnight at 4°C. To stain nuclei, Hoechst solution (34580, Invitrogen) or DAPI (ab228549 , Abcam) was added at 1:1000 in 0.25% Triton, 1% BSA in PBS for 4 hours at room temperature after the incubation of secondary antibody.

The primary antibodies used to mark the lumen were anti-Aurora B (Becton Dickinson), anti-Ezrin (3C12) (sc-58758, Santa Cruz), anti-Mucin-1 (MH1(CT2)) (MA5-11202, Thermofisher Scientific), anti-ZO1(1A12) (339100, ThermoFisher Scientific), anti-aPKC (H1) (sc-17781, Santa Cruz), and Alex-488 Phalloidin (A12379, Thermofisher Scientific). To mark epithelial cells anti-Ecad (M108, Takara Bio), and anti-Sox9 (AB5535, Merk) were used. To mark proliferating cells, anti-phosho Histone-3 Serine-10 (3H10) (05-806, Millipore/Sigma Aldrich) was used. Secondary antibodies used were goat anti-Armenian hamster IgG H&L (Alexa Fluor 568) (ab175716, Abcam), goat anti-Mouse IgG H&L (Alexa Fluor 488) preadsorbed (ab150117, Abcam), anti-Mouse IgG H&L (Alexa Fluor 647) preadsorbed (ab150111, Abcam). Primary and secondary antibodies were used with a dilution factor of 1:400 for all immunofluorescence experiments.

0.5 Clostridium Perfringens enterotoxin (CPE) fragment expression, purification and labelling

0.5.1 Cloning

The non cytotoxic, claudin binding, C-terminal domain of CPE was been expressed and purified according to Tachibana *et al.* with few modifications [36]. In details, codon optimized cCPE fragment, amino acids 184-319, sequence: ERCVLTVPSTDIEKEILDLAAATERLNLTDALNSN PAGNLYDWRSSNSYPWTQKLNLHLTITATGQKYRI LASKIVDFNIYSNNFNNLVKLEQSLGDGVKDHYVD ISLDAGQYVLVMKA NSSYSGNYPYSILFQKF (Twist Biosciences) was cloned into p7XNH3 vector and tagged with a N-terminal 10xHis tag cleavable with human rhinovirus 3C protease [37].

0.5.2 Expression

E.coli T7 Express strain (New Englad Biolabs) was transformed with p7XNH3-10xHis-3C-cCPE and pRare plasmids. A preculture was grown in LB media supplemented with 1% glucose, 30 μg/ml kanamycin (kan) and 17 μg/ml cloramphenicol (cm), overnight at 37°C, shaking at 150 rpm (Kunher shaker). E.coli cultures for induction were grown in Terrific broth (TB), supplemented with 90 μg/ml kanamycin and 17 μg/ml chlorampphenicol antibiotics at 37°C. When the optical density at 600 nm reached a value of 0.6-0.8, cultures were moved into a 18°C shaking incubator. Protein expression was induced with 0.2 mM IPTG (Sigma) final concentration, overnight at 18°C.

0.5.3 Lysis, IMAC, His tag removal and Size Exclusion Purification Steps

E.coli were harvested by centrifugation at 6,000 g, for 10 min, at 4°C (JLA 8.1000 rotor, Beckman), lysed in Lysis buffer (20 mM Hepes, 0.5 M NaCl, 2 mM MgCl2, pH7.2, 5% glycerol, 1 mM DTT) containing Protease Inhibitors EDTA-free (Bimake) and Benzonase (Merck), with a high pressure homogenizer LM-20 (Microfluidics), using two passages at 20,000 psi. Insoluble material was removed by high speed centrifugation (30,000g, 1 hour, at 4°C, in rotor JA12, Beckman) and by 0.45 μm filtration. IMAC purification was performed with 5 ml HisTrap FF columns (Cytiva). After equilibration and loading, the column was washed with Lysis buffer, supplemented with 20 and then 50 mM imidazole. Finally, 10xHis-tagged-cCPE was eluted with IMAC elution buffer (20 mM Hepes, 0.5 M NaCl, pH7.2, 5% glycerol, 500 mM imidazole, 0.5 mM TCEP). To remove the 10xHis tag, the protein was incubated with HRV3C protease (Merck) while dialysed against a size exclusion buffer (20 mM Hepes pH7.2, 300 mM NaCl, 5% glycerol, 0.5 mM TCEP) overnight at 4°C. Size exclusion chromatography was performed with HiLoad Superdex200 column (Cytiva), equilibrated in 20 mM Hepes pH7.2, 300 mM NaCl, 5% glycerol, 0.5 mM TCEP.

0.5.4 cCPE labelling with ATTO647

cCPE was labelled with ATTO647 maleimide (Sigma), according to the manufacturer's protocol. Labelled cCPE was separated from free dye excess using first a desalting nap-5 column (Cytiva), followed by gel filtration, using a 24ml Superdex75 column (Cytiva) equilibrated in 20 mM Hepes pH7.2, 300 mM NaCl, 5% glycerol, 0.5mM TCEP. cCPE-labelled fractions were analysed by SDS PAGE. Positive fractions were pooled and protein concentrated. The degree of labelling was calculated to be 0.6.

0.6 Viscosity estimation with 3D particle tracking

To estimate viscosity in approx. 90% H_2O and approx. 1% glycerol, Carboxyl Fluorescent Pink Particles (CF-2058-2, Spherotech, Inc.) was used at 1:10 dilution. To estimate viscosity in the lumen, spherical organoids were treated with Cell-Mask-orange (C10045, Invitrogen) at a concentration prior to image acquisition (Supplementary Figure 2a). Following approx. 2 hours of treatment CellMask-orange positive particles were imaged in a Spinning-disk microscope. A 3D stack imaging was performed with a z resolution between 0.25-0.35 µm and time-resolution of 0.7-1.2 sec depending on the size of the 3D stack at a given time point.

To obtain particle segmentation & trajectories, images were denoised by using Noise2Void [38]. Afterwards, particles were manually cropped in 3D and time. To segment the particles, a combination of StarDist and APOC (Accelerated Pixel and Object Classification) python-based tools were used to obtain 3D labels in time [39, 40]. To obtain particle trajectories, LapTrack & Napari-Laptrack were used to track particles according to their distance and image-based features (intensities and size) between time frames (Supplementary Figure 2b) [41].

For the estimate of the diffusion coefficients of the particles, trackpy was utilized to obtain 3D mean squared displacement curves from the particle trajectories obtained [42]. To obtain accurate diffusion coefficients, particles with tracks longer than 20 time frames and a regression of above 0.75 for the linear fit of the mean squared displacement were selected. To measure the size of particles, the biggest area (in the z-axis of the segmentation) was used to derive the radius of the particle. As a result, we

observed that the particles had a negative correlation between particle radius and diffusion coefficients (Supplementary Figure 2e & f). To estimate the viscosity of fluids using the obtained particle radius and diffusion coefficients, we used the Stokes-Einstein equation (Supplementary Figure 2c).

0.7 Inference of hydrostatic lumen pressure with linear laser-ablation

First, the organoids were taken out of the Matrigel to perform the laser ablation. This was achieved by mechanically breaking the Matrigel dome (with organoids within) with pipettes followed by Liberase (CF-2058-2, Spherotech, Inc.) incubation at 37°C for 15 min to achieve enzymatic dissociation. Afterwards, individual organoids were transferred onto 8 well glass-bottom plates (80826,Ibidi) containing organogenesis medium w/o chemical treatments.

To create conduits accross the epithelium for inferring hydrostatic pressure of the lumen [8], a laser-ablation was performed by utilizing a Zeiss LSM 780 NLO system (Zen Black v11.00 software) with a 2-photon laser (Titatnium/Saphire). With the Zen Black software the 2-photon laser, with a power of 3.2 W at the laser head, was set to 100% laser power at a wavelength of 800 nm. A line scan with a width of 6.6-9.9 µm and 40–50-line repetitions across the epithelium at the middle plane of a lumen was performed to create a cut across the epithelium. Prior and post laser cutting, a 3D stack of the lumen was imaged (with a time resolution of 10-25 seconds: depending the size of the lumen) to later measure the lumen volume changes.

FiJi software was used to quantify the image-based variables for the Hagen-Poiseulle model. The line-tool and measure function was used to measure the monolayer thickness and radius of the channels (created by the laser ablation) [43]. LimeSeg, a Fiji plugin for the segmentation of 3D objects, was used to quantify the flow rate via lumen volumes changes before and after the laser-ablation [44].

0.8 Epithelial permeability assay

As a readout for epithelial permeability of pancreatic organoids, 3-5kDa Dextran-Alex488 (D22914, ThermoFisher Scientific) or 10kDa Dextran-Alex647 (D22914, ThermoFisher Scientific) were supplemented to the organoid media. After 1-3 hours of incubation, organoids were imaged with a spinning-disk microscope and light-sheet microscope.

0.9 Transcriptome Analysis

Branching and spherical organoids at day 7 of culture were lysed with lysis buffer RLT, and RNA was purified following the manufacturer instructions (RNeasy Plus Micro Kit, 74034, Qiagen). The quality of the purified RNA using an Agilent 2100 Bioanalyzer, following the instructions of the manufacturer (Agilent RNA 6000 Pico Kit 5067–1513). Amplification of the extracted RNA (700 pg) was performed by Ovation Pico SL WTA system V2 (3312–48, Nugen). Samples were labeled with SureTag DNA labeling kit (5190–3391, Agilent Technologies), run on SurePrint G3 Mouse Gene Exp v2 Array (G4852B, Agilent Technologies), and signals were read by a SureScan Microarray Scanner (Agilent Technologies).

0.10 EdU incorporation assay

Organoids were incubated with 10 μM EdU (Click-iTPlus EdU Alexa Fluor 647; C10640, Invitrogen) in organogenesis medium for 2 hours at 37°C and 5% CO_2. Then, organoids were processed for immunostaining as described above. Permeabilisation, blocking, and Click-iT reaction for EdU detection were performed according to the manufacturer's instructions.

0.11 Microscopy

0.11.1 Spinning-disk microscopy

All spinning-disk microscope images were performed with a Olympus IX 83 inverted stand driven by the Andor iQ 3.6 software. The microscope is equipped with a motorized xyz stage, a Yokogawa CSU-W1

scan head, and an Olympus 30 × 1.3 NA Sil Plan Apo objective. The setup was located inside a temperature-controlled chamber set to 37°C and 5% CO2. The sample was illuminated with a 561 nm laser, and the emission was collected using a 525/50 bandpass filter.

0.11.2 Confocal microscopy

All confocal images were acquired using an inverted Zeiss LSM 780 confocal microscope with a 40×/1.3 NA C-Apo water objective, 561 nm diode laser, and a pinhole setting between 1 and 2 Airy Units. All images were acquired using Zen Black software (Carl Zeiss).

0.11.3 Light-sheet microscopy

Organoids were grown in V-shaped bottom chambers made from Fluorinated ethylene propylene (FEP) from Viventis. A Viventis light-sheet microscope was used with a Nikon 25x 1.1 NA water immersion objective. The imaging parameters were set to: dual illumination of 2.2 μm beams with Laser 561/647, and an exposure of 100 ms. The chamber was set to 37°C and 5% CO2. Organoids were imaged every 30 min intervals.

0.12 Image analysis and Quantification

0.12.1 2D & 3D organoid and lumen segmentation and quantification

To segment the lumen and whole organoid structure, images were first denoised using Noise2Void [38]. Images containing epithelial markers (nuclei and membranes) were summed using pyclesperanto-prototype [45]. The summed epithelium channel was then processed with Gaussian blur (sigma for xyz axes = 0.75-1.5) and Top-hat background removal (radius for xyz axes = 20-30). These processed channels, along with the lumen within the epithelium, were manually annotated using Napari to create training data for an APOC model [39]. Using the trained APOC model, the epithelium channels were segmented. Inaccuracies in the prediction output were manually corrected with Napari or semi-automatically corrected using the binary processing functions of pyclesperanto-prototype. The lumen, identified as a 3D hole in the epithelium mask, and the segmentation output were used to generate triangulated meshes. To perform 2D segmentation of organoids and lumen structures, the largest area along the z axis was selected from the 3D segmentation output for further analysis and quantifications.

To generate meshes from the lumen and epithelium, 3D Marching-Cube function of scikit-image were applied on the lumen binary and the epithelium binary, that had been processed with the binary fill holes function of scipy-image and rescaled pixel of pyclesperanto-prototype for isotropic pixels [45, 46]. The generated meshes were smoothened using the Laplacian smoothening fucntion of Trimesh [47]. Other features of the lumen and organoid meshes were obtained via Trimesh functions: integrated mean curvature, volume, and surface area. To generate the topological skeleton of the lumen, the Skeletonizing function of the scikit-image were applied to the lumen binary [46].

The following calculations were performed to obtain the morphological features of the lumen and organoids:

- **Lumen and organoid sphericity**: to numerically characterize the 3D shape of the objects we quantified the reduced volume (v) by applying the volume (V) and surface area (SA) obtained from the generated meshes (above) [10]. This quantification resulted in perfect spheres exhibiting a reduced volume of 1 and in lower values with decreasing sphericity.

$$Sphericity = 3\sqrt{4\pi}\, V/SA^{3/2}$$

- **2D and 3D Lumen occupancy (LO)**: to obtain the 3D lumen occupancy, volumes (V) obtained from the 3D segmentation of the lumen and organoid we used. For 2D lumen occupancy, areas (A) obtained from the 3D segmentation of the lumen and organoid we used.

$$3D\, Lumen\, occupancy = V_{Lumen}/V_{Total}$$

$$2D\,Lumen\,occupancy = A_{Lumen}/A_{Total}$$

The lumen occupancy values are presented as percentages, except for Supplementary Figure 4.

0.12.2 3D nuclei segmentation and quantification

The segmentation of nuclei in 3D-images was performed using StarDist [40]. First, a subset of images with nuclei staining were manually annotated using Napari as training data to create a StarDist model. After, the trained model was applied to predict and segment the nuclei. The nuclear segmented output was used to quantify the number of EdU-, DAPI-, and Hoescht-marked nuclei in organoids.

The following calculations were performed to obtain the proliferation features of the organoids:

- **EdU:DNA ratio**: To quantify active proliferation detected with the EdU incorporation assay we obtained the total number of EdU and DNA per organoid from the nuclear segmentation (above). From that we presented the data as ratio

$$EdU : DNA\,Ratio = Count_{EdU}/Count_{DNA}$$

- **Doubling time**: To quantify the rate of cell population doubling we obtained the average number of cells at 48 hours ($\overline{N}_{48}$) and 96 hours($\overline{N}_{96}$)

$$Doubling\,time(hours) = \frac{Duration \times \ln(2)}{\ln \frac{\overline{N}_{96}}{\overline{N}_{48}}}$$

0.13 Mathematical model

To computationally simulate the multicellular morphology, we applied the multicellular phase-field model [48] with lumen phase [21, 49]. Although the details of the model used in this paper follow those in Tanida *et al.* [21], we changed the cell growth rule in the following way. In Tanida *et al.*, we assumed the term $\alpha[V_{target} - V_i(t)]$ in the free energy functional with the constant target volume V_{target} as well as the variable area of each (i-th) cell $V_i(t)$ and the constant prefactor *alpha*; in contrast, in this paper, this term is replaced by $\alpha[V_{target,i}(t) - V_i(t)]$ where $V_{target,i}(t)$ is the time-dependent target volume, which allows us to control the cell growth rule. The time evolution of $V_{target,i}(t)$ was assumed to obey

$$\tau_V \frac{dV_{target,i}(t)}{dt} = \bar{V} - V_{target,i}(t) \tag{1}$$

(*i.e.* exponential convergence toward the given constant $\bar{V}$ with the given characteristic convergence time τ_V), so that we can control the typical cell growth duration by tuning the parameter τ_V. Finally, in this paper, we do not assume the minimum duration for the division of each cell after the division of its mother cell, which we assumed in Tanida *et al.* Parameter values used in this paper are as follows; $\alpha = 1.0$, $\bar{V} = 3.0$, with $\tau_V = 1, 10, 20, 30, 40, 50, 60, 70, 80, 90$ and ξ varying from 0.14 to 0.34 with increments of 0.02. The other parameter values and the initial conditions are set identical to those of Tanida *et al.*

Growth of the lumen volume is governed by the hydraulics of the lumen and cell layers [50, 51] and the dynamics of lumen volume in our model is also controlled by the similar equation as

$$\frac{dV_0}{dt} = \lambda_w A_{apical}(\Pi - \Delta p) = \lambda_w A_{apical}\left(\frac{\xi}{6} - \frac{2T}{R}\right), \tag{2}$$

where λ_w is the water permeability of the membrane. A_{apical} is the area of the apical side of the monolayer. T is the tension of cell layer surrounding the lumen. R is the radius of the spherical lumen. Note that the first equality is often used in the hydraulics of lumen dynamics, where Δp is the hydrostatic pressure difference between lumen and outside the cyst. The second equality can be derived analytically

from the dynamics of phase field model (PFM) for an isolated domain (here we refer to it as lumen) with T the surface tension of the interface of the lumen, and was successfully tested numerically [21]. When the lumen is surrounded by a cell layer, the tension of the cell layer is added to interfacial tension of the lumen, bringing a relation, $\Delta p = 2T_{total}/R$, with $T_{total} = T + T_{cell}$. In order to test the relationship between the osmotic pressure Π and Δp, we performed a growing spherical cyst simulation by varying ξ. By assuming that $\lambda_w A_{apical}$ is the constant and measuring dV/dt with varying ξ, Δp can be estimated at each value of ξ. The result shows that Δp is proportional to ξ with an offset which determines the critical pressure for lumen growth. It manifests that $\xi \sim 0.7\Pi$ (in non-dimensional unit).

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