Long-term culture of human pancreatic slices as a model to study real-time islet regeneration

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The culture of live pancreatic tissue slices is a powerful tool for the interrogation of physiology and pathology in an in vitro setting that retains near-intact cytoarchitecture. However, current culture conditions for human pancreatic slices (HPSs) have only been tested for short-term applications, which are not permissive for the long-term, longitudinal study of pancreatic endocrine regeneration. Using a culture system designed to mimic the physiological oxygenation of the pancreas, we demonstrate high viability and preserved endocrine and exocrine function in HPS for at least 10 days after sectioning. This extended lifespan allowed us to dynamically lineage trace and quantify the formation of insulin-producing cells in HPS from both non-diabetic and type 2 diabetic donors. This technology is expected to be of great impact for the conduct of real-time regeneration/developmental studies in the human pancreas.

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The identification of methods to stabilize, section and culture slices of live pancreatic tissue has represented a qualitative leap in our ability to study the biology and function of the pancreas. This in vitro setting preserves much of the cytoarchitecture of the organ, allowing for the dynamic study of islet physiology and the interactions between endocrine, exocrine, neural, vascular and immune cells in their anatomical context. Since this method is less damaging to the tissue than those used to isolate islets or exocrine tissue, the studied phenomena are more likely to approximate the native biology of the organ. As originally reported, the method entails the retrograde injection of low melting point (LMP) agarose through the major pancreatic duct of mice. LMP hardens and stabilizes the pancreas, which can be subsequently extracted, further embedded in agarose and sliced with a vibratome. Slicing techniques have recently been adapted to human pancreas. These reports have attracted widespread interest, especially owing to simultaneous initiatives such as that of the Network of Pancreatic Organ Donors with Diabetes (nPOD) program, to obtain and distribute live pancreatic tissue from type 1 diabetes (T1D) patients. The use of slicing techniques on those precious samples has the potential to unveil a wealth of information on the dynamic processes of autoimmunity and regeneration in T1D. However, current settings (which entail the culture of slices atop transwell inserts) have not been tested for long-term culture, a sine qua non requirement for the study of regeneration. Based on our previous experience with human isolated islets and embryonic-stem cell (hESc)-derived β-like cells, we anticipated that improper oxygenation might induce hypoxia and thus compromise HPS viability and function over several days of culture. This was confirmed experimentally through metabolic and viability assays. Therefore, we sought to identify improved ways to culture HPSs long-term. Using a perfluorocarbon (PFC)-based system designed to enhance oxygen transfer to three-dimensional tissues, as well as a refined medium composition, herein we present evidence that HPSs from both healthy and T1D/T2D donors survive and exhibit normal function beyond 10 days of culture. This extended lifespan was sufficient to detect, longitudinally track and quantify β-cell regeneration in slices from transgenic mice as well as HPSs from both healthy and T2D human donors co-transduced with adenoviral vectors carrying a reporter and an insulin lineage tracer. The latter approach opens the door to the study of human pancreatic remodeling and regeneration in an in vitro setting that largely preserves the compartment distribution, cell-to-cell interactions and niches of the native organ.

Results
Mathematical modeling of oxygen diffusion in HPSs. To predict oxygenation patterns across 120 µm-thick HPSs, we performed 2D diffusion reaction modeling of oxygen distribution using COMSOL v5.3 (COMSOL Inc, Burlington, MA) as described. A parametric sweep of oxygen consumption rate (OCR) (1.0–5.0 x 10^-2 mol/m^3 s^-1) unique to pancreatic tissue was performed. An estimated oxygen consumption rate (OCR) of 0.03 mol/m^3 s^-1 (average for islets and acinar tissue) was used (Fig. 1). Two culture settings were modeled: standard transwell and PFC-based dishes. While an air-liquid interface above the tissue is acceptable for short-term analyses, the medium is impermeable to oxygen transport to three-dimensional tissues as oxygen concentrations drop inside the slices after 24 h. Therefore, we proceeded to culture HPSs in transwells and PFC dishes for 10 days. The average OCR in the transwell group was 21.9 ± 8.3 pm/µg of protein, considerable higher in the transwell group vs. PFC, both overall (AUC p-value = 0.03) and at every time point analyzed (Fig. 1e). Also, as predicted, HPSs cultured in transwells displayed a higher glucose consumption rate than those cultured in PFC counterparts at every time point after day 1 (AUC p-value = 0.04) (Fig. 1f), an observation that likely reflects a heightened need of sugar to keep up with energy demands in partially anaerobic conditions. We further measured ATP production for n = 3 slices cultured in either TW or PFC for 10 days. The average ATP production in transwell-cultured slices was 21.9 ± 8.3 pm/µg of protein, compared to 57.7 ± 42.5 pm/µg in those cultured in PFC, which is statistically insignificant (p-value = 0.06) (±, standard deviation, twailed t-test). However, the production of ATP (pm)/µg of glucose, normalized by µg of protein, was 38.5 in the PFC group vs. 6.8 in the TW group (a ~6-fold increase). Thus, while both groups have comparable production of ATP, slices cultured in PFC dishes generate it with six times less glucose. Figure 1g shows the higher relative uptake of the fluorescent D-glucose analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) by slices cultured in transwells vs. those in PFC during a 10-minute incubation. Quantification of the mean fluorescence intensity (MFI)/DAPI+ cells in both groups (0.53 ± 0.15 for the transwells vs. 0.075 ± 0.06 in PFC, a 7-fold increase)
per Fig. 1 Enhanced oxygenation of human live pancreatic slices prevents anoxia and preserves oxidative phosphorylation. a Diagram outlining the perfluorocarbon (PFC) membrane AirHive cell culture dish compared to a transwell dish. PFC dishes are selectively permeable to gases but not liquids. Therefore, medium can be added from the top while air is preferentially transferred directly from the bottom. Graphics partially done with BioRender.

b COMSOL modeling of oxygen partial pressure across human pancreatic slices cultured in transwells (left) or PFC membranes (right) over a 24 h period. Spectral scale ranges from high (red) to low (blue) oxygen partial pressure. Anoxic areas are in white. 

b Therefore, medium can be added from the top while air is preferentially transferred directly from the bottom. Graphics partially done with BioRender.

c Glucose consumption rates of PFC- (red) and transwell- (blue) cultured slices as a function of time. The dashed threshold represents anoxia. 

d Total anoxic volume of PFC- (red) and transwell- (blue) cultured slices modeled against increasing oxygen consumption rates. 

e HIF-1α protein level determination in transwell- vs. PFC- cultured slices after 24 h of culture (n = 3 biologically independent samples from individual donors). Two-tailed t-test: *p-value = 0.0304, f Lactate production rate (μg/μg DNA) of PFC- (red) and transwell- (blue) cultured slices as a function of time. n = 3 biologically independent samples from individual donors. Multiple two-tailed t-tests: **p-val3days = 0.0083, **p-val5days = 0.0043, **p-val7days = 0.0037, **p-val10days = 0.0063, **p-val14days = 0.0192, ***p-val16days = 0.0092, ***p-val21days = 0.0004, ***p-val30days = 0.0009. 

g Glucose consumption rates of PFC- (red) and transwell- (blue) cultured slices as a function of time. n = 3 biologically independent samples from individual donors. Two-tailed t-tests: **p-val1day =0.0063, *p-val3days = 0.0176, **p-val6days = 0.0244, *p-val9days = 0.0343. h Representative immunostaining of 2-NBDG uptake (green) during a 10-min incubation of human pancreatic slices cultured in transwells (top) or PFC dishes (bottom), reflecting the higher relative uptake of glucose of the former. Red: Propidium Iodide (PI), indicative of cell death. Blue: DAPI. Scale: 100 μm. 

b-d represent theoretical computational modeling and have no error bars. In e, data are presented as mean ± S.E.M; in f, g, h as mean ± SD. Each n further represents the mean of three technical replicates. Source are provided in the Source Data file.

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confirms that the differences between the two groups are statistically significant (two-tailed t-test, p-value = 0.02). The lower glucose uptake in the PFC group was not due to lower viability of the tissue. In fact, the opposite is true, as there was a 2.6-fold increase in the ratio of propidium iodide (PI, cell death marker); DAPI in the transwell (0.40 ± 0.04) vs. the PFC (0.14 ± 0.06) group (two-tailed t-test, p-value = 0.007). This observation further supports the hypothesis that slices cultured in transwells require higher glucose uptake.

While carbon isotope techniques would be necessary to conclusively demonstrate metabolic changes, our data collectively suggest that culture of HPSs in PFC membranes preserves the usage of physiological oxidative phosphorylation as a primary source of energy over an extended period.

Long-term viability of HPSs cultured on PFC-based membranes. Decreased viability and tissue degradation are expected outcomes of sub-optimal oxygenation. We hypothesized that PFC-mediated enhanced oxygenation, coupled with proteolytic enzyme inhibition, would improve the overall long-term viability of HPSs vs. standard culture. To test this, we proceeded to culture HPSs from n = 5 independent (non-T1D) donors. (demographics in Supplementary Table 1). For each donor, n = 20 slices were assigned either to PFC (n = 10) or transwell (n = 10) groups. Consecutively cut slices were split between the two groups to minimize the impact of potential regional variations. Figure 2a shows live (green)/dead (red) imaging of a representative freshly sectioned slice, typically >80% viable (Fixed Viability Dye, Thermo-Fisher Scientific, Waltham, MA). A dead (fixed) slice is stained for comparison in Fig. 2b. Following extended culture, the viability (percentage of live/dead cells) (Fig. 2c–e, Supplementary Fig. 1b) and cross-sectional area (reflective of overall tissue integrity) of HPSs cultured in transwells are lower than those observed in PFC (Fig. 2f–g, Supplementary Fig. 1c). Slices from a T1D donor (Supplementary Table 1) also exhibit the same behavior when kept in either culture setting, as indicated in Fig. 2h–i. To further characterize the effects of culture conditions on HPSs, we performed immunofluorescence assays for endocrine and exocrine markers. Figure 2j shows rounded islets and well-preserved islet endocrine cell types both at day 0 and at day 10 of PFC culture. However, those of HPSs kept in transwells have less-defined boundaries (Fig. 2j).

All the quantified endocrine cell types were positive for their respective hormone and DAPI, suggesting nuclear integrity. The relative proportion of β-, α-, and δ-cells remains unchanged (Fig. 2k) in all conditions. This observation, coupled with the overall reduction in cross-sectional surface (Fig. 2d, e) and smaller islet area in transwells at 10 days (Fig. 2l) suggests that progressive cell death in transwells occurs similarly across all cell populations. Otherwise, the relative abundance of Ki67-positive cells is similar in both groups at day 10, with most proliferative cells observed in the exocrine compartment (Supplementary Fig. 2a). Therefore, culture in PFC dishes does not induce apparent changes in cell turnover throughout a 10-day period.

Examination of key β-cell transcription factors NKX6.1 and PDX1 at day 10 indicates that their expression is stronger in PFC than transwell conditions (Supplementary Fig. 2b, c). While no differences in amylase signal were apparent at low magnification between day 0 and day 10 on either condition (Fig. 2m), higher magnification images show that PFC-cultured slices have well-preserved E-cadherin ultrastructure and display the typical concentration of granules in the apical region of the cells (Supplementary Fig. 2d, bottom). In contrast, slices cultured for 10 days in transwells have less-defined E-cadherin and acinar morphology, with more diffuse amylase staining (Supplementary Fig. 2d, top). This resolution was insufficient to detect potential ultrastructural changes such as increase vacuole formation, basal membrane abnormalities or junctional complex disruption.

qRT-PCR was conducted to test whether the expression of key endocrine and non-endocrine genes was affected by long-term culture in PFC- vs. transwell-cultured slices. As shown in Supplementary Fig. 3, all the examined markers are upregulated in PFC vs. transwells, further suggesting that the former preserves better the integrity and function of HPSs.

Enhanced oxygenation supports extended function in HPSs. The claim that HPSs sustain function throughout long-term culture must be substantiated by functional assays for both the endocrine and exocrine compartments. To do so, we measured dynamic glucose-responsive insulin secretion (perifusion) and glucose-mediated calcium imaging (both for endocrine activity), as well as carbachol (CCh)-mediated release of digestive enzymes indicative of exocrine function. Figure 3a shows the pattern of glucose-dependent insulin release (perifusion assay) in live slices shipped from nPOD’s Gainesville laboratory (day +1S, one day after sectioning, shipped; black), day +10 transwell (blue) and day +10 PFC (red) islet-containing HPSs from n = 3 independent donors. Perifusion charts for each donor are shown in Supplementary Fig. 4. Figure 3d presents the analysis of HPSs from a short-term T1D donor. Stimulation indices (SI) normalized to baseline are in the Y-axis, with areas under the curve (AUC) values in Fig. 3b, c (for non-diabetic donors) and Fig. 3e, f (for the T1D donor).

All three groups (day +1S, day +10 transwell and day +10 PFC) exhibit glucose-stimulated responsiveness, although typical biphasic responses were not observed. However, in comparison with the PFC group, acute (i.e., shipped from nPOD) and transwell groups showed decreased insulin secretion at 16 mM glucose and 30 mM KC1 stimulation. SIs were higher in PFC than in transwells, and differences were statistically significant (Fig. 3c).

Interestingly, acute samples (day +1S) normally had poorer glucose responsiveness than those analyzed at day 10, which may reflect shipment stress. This mirrors the reduction of islet function immediately after isolation. In fact, islets are typically allowed to rest for 24–48 h prior to the conduct of functional assays, a period during which they deactivate stress signaling pathways22,23. To test whether resting the slices for 24 h after being subjected to stress would lead to functional recovery, we conducted additional experiments on n = 3 new donors. In this case, HPSs were generated in-house and perfused immediately after slicing (day 0), after 24 h recovery (day +1) and then at day +10 (transwells or PFC dishes). We hypothesized that day 0 HPSs would be stressed in the perisecting period, thus having poorer responses than those allowed to rest an additional day. This hypothesis proved correct, as evidenced by a significant rebound in the SI of HPSs rested for 24 h (Fig. 3g–i). Of note, day 0 responses in this setting are roughly equivalent to those observed at day +1S in Fig. 3a, suggesting that both forms of stress (shipping and slicing) have a deleterious, yet transient effect on function. The glucose responsiveness of slices in PFC was higher than that recorded in the transwell group at day 10, but in this particular set of experiments (unlike in the previous one) the differences were not statistically significant. This is probably due to the high donor-to-donor variability, as shown in Supplementary Fig. 4b (individual donor perifusions) and Supplementary Fig. 3 (qRT-PCR). All the experiments subsequently presented showing acute measurements were conducted on rested slices.
**Fig. 2 Human pancreatic slices remain viable after extended culture.**

- **a**: Representative immunostaining showing tissue viability at the time of tissue slicing, showing live (green) and dead (red) regions. Scale: 500 μm for all panels. **b**: Immunostaining of control fixed slice showing only dead cells. Scale: 500 μm.
- **c**: Immunostaining showing slice viability assessments after 10 days of culture in either transwells or PFC dishes. Necrotic zones in transwell-cultured slices are highlighted in magnified panel. Overt necrosis, in contrast, is not apparent in PFC-cultured HPSSs. Scale: 500 μm. Magnified insets: 200 μm. For **a–c**: n = 5 biologically independent samples from individual donors.
- **d**: Quantitative measurement of viable cell percentages in PFC (red)- and transwell (blue)-cultured slices over a period of 10 days. Shaded area shows mean ± SD.
- **e**: Bar plots showing cellular viability of either culture setting at day 5 and 10 post-slicing. Two-tailed t-test: *p-val10days ≠ 0.002.
- **f**: Slice cross-sectional area of slices kept in either culture condition for 10 days in culture. Shaded area shows mean ± SD.
- **g**: Individual bar plots showing slice cross-sectional area at days 7, 14, and 21 post slicing. Two-tailed t-test: **p-val14days = 0.0036, ***p-val21days = 0.000087. For **d–g**: n = 4 biologically independent samples from individual donors.
- **h**: Viable cell percentages of PFC- and transwell-cultured T1D donor slices over a period of 10 days (n = 1 sample from an individual donor).
- **i**: Slice cross-sectional area in either culture condition over 10 days of culture (T1D donor) (n = 1 sample from an individual donor).
- **j**: Immunofluorescence images showing expression of representative endocrine markers (INS insulin, white; GCG glucagon, red; and SST somatostatin, green) within islets of slices at day 0 and cultured for 10 days in either transwells or PFC dishes. Scale: 500 μm. **k**: Dot plot showing the distribution of endocrine cells in islets at day 0 and after 10 days of culture (PFC and transwells). Two-tailed t-test: *p-val0days ≠ 0.0107. For **j–k**: data from n = 10 biologically independent samples from individual donors.
- **m**: Immunostaining showing insulin (INS, white) and representative exocrine markers (cytokeratin-19, CK-19, red; and amylace, AMY, green) within slices at day 0 and after 10 days of culture (PFC and transwells). Scale: 500 μm; 100 μm for insets. For **j–m**: data are presented as mean ± SD. Each n further represents the mean of three technical replicates. *p < 0.05; **p < 0.01; ***p < 0.005. Source are provided in the Source Data file.
As Ca^{2+} imaging reflects β-cell activity\textsuperscript{24}, we set out to determine whether HPSs cultured for 10 days in either condition exhibited changes in cytoplasmic Ca^{2+} concentration in response to glucose stimulation, using the calcium indicator Fluo-4AM. This technique allows to measure β-cell activity in response to secretagogues such as 16.7 mM glucose or 30 mM KCl (Fig. 3m, n and Supplementary Movie 3). Select β-cells were observed to...
respond (increased cytoplasmic Ca$^{2+}$) to 16.7 mM glucose as well as 30 mM KCl in HPSCs cultured for 10 days atop both transwells and PFC membranes. KCl responses were not significantly different between day 10 PFC- and transwell-cultured HPSCs. However, only PFC-cultured HPSCs showed a significant difference between baseline (3 mM) and stimulation concentrations (16.7 mM) of glucose (Fig. 3o–p). We found β-cells to be synchronous and oscillatory in both groups, without disruption in coupling. Overall, pulses in cultures transwell-cultured slices during 16.7 mM glucose stimulation were weaker than those in PFC, but the results were not significantly different.

Exocrine cells respond to acinar-specific secretagogues such as carbachol (acetylcholine agonist) and cholecystokinin$^{45}$ in this respect, we observed that exocrine cells in HPSCs responded to exocrine function of total slice area longitudinally. When this experiment was repeated using slices treated with the β-cell toxin alloxan, control (non-BMP-7-treated) slices displayed virtually no green signal. The red signal, corresponding to reporter-transduced slices, was similar for all the experiments (Fig. 4g), indicating no aberrant transduction efficiency due to alloxan treatment. However, when the slices were treated with BMP-7, green signal was progressively detected after alloxan administration, demonstrating new insulin$^{+}$ cell formation (Fig. 4i–k). Partially confirming the β-cell

Perifusion of HPSCs after high glucose (16.7 mM, 16 G) and KCl (30 mM) stimulation. a: Amylase secretion after 10 μM CCK8 stimulation. Middle, AUC for 3 mM glucose stimulation with 100 ng/ml of BMP-7. Control slices received vehicle instead of BMP-7. From day 6–9, BMP-7 was no longer administered. As shown in Fig. 4b, newly formed insulin$^{+}$ cells were observed starting at day 9 in regions that had been previously devoid of green (insulin) signal. No such occurrence was detected in controls. Figure 4c presents another similarly designed experiment using a BMP-7-like agonist, THR-12327,29. In this case, green cells were detected from day 7, mostly in a region corresponding to a large pancreatic duct.

To see whether we could replicate this model in non-transgenic mice, we co-transduced pancreatic slices from CD-1 (wild-type) mice with adeno-viruses carrying the reporter construct CMV-loxP-dsRED-loxP-EGFP (kindly given by P. Ravassard) and a rat insulin promoter (RIP)-driven Cre recombinase (Fig. 4d). We predicted that doubly transduced non-β-cells would be tagged red and pre-existing β-cells would be tagged green. As in the previous transgenic setting, an additional prediction was that doubly transduced insulin$^{+}$ cells arising from non-β-cells would be transiently tagged red with decaying dsRED and permanently green with newly produced EGFP (transient yellow). We treated slices with BMP-7 as above. In all, 24 h after the double transduction, islets were labeled green, as shown in the lower-right inset of Fig. 4e. The formation of new insulin-expressing cells from non-β-cells was confirmed in several regions of the slices, normally associated to ductal areas, where groups of red cells progressively transitioned to green following the treatment with and subsequent withdrawal of BMP-7 (Fig. 4e). Red and green signal could be readily monitored and quantified as a function of total slice area longitudinally. When this experiment was repeated using slices treated with the β-cell toxin alloxan, control (non-BMP-7-treated) slices displayed virtually no green signal throughout (Fig. 4f–h). As an additional internal control, non-alloxan-treated slices (dashed-green line, Fig. 4h) exhibit readily detectable green signal (corresponding to islets). The red signal, corresponding to reporter-transduced slices, was similar for all the experiments (Fig. 4g), indicating no aberrant transduction efficiency due to alloxan treatment. However, when the slices were treated with BMP-7, green signal was progressively detected after alloxan administration, demonstrating new insulin$^{+}$ cell formation (Fig. 4i–k). Partially confirming the β-cell
Fig. 4 Tracking of β-cell regeneration in murine pancreatic slices. a Representative microphotograph of murine pancreatic slices from Ins2-Cre.mTmG mice, with EGFP+ insulin-producing cells (green) and all other cells tdTomato+ (red). An islet and adjacent duct are magnified in the inset. Scale 300 μm. 
b Following 5 days of BMP-7 stimulation, removal thereof leads to insulin+ cell formation at d9. Scale 300 μm (magnified insets: 50 μm). c Representative microphotograph of murine slices from Ins2-Cre.mTmG mice treated for 7 days with THR-123. Green arrow shows a pre-existing islet. After THR-123 removal, new insulin+ cells are EGFP-tagged within the β-cell formation at d9. Scale 300 μm (magnified insets: 50 μm). c Representative microphotograph of murine pancreatic slices from Ins2-Cre.mTmG mice treated for 7 days with THR-123. Green arrow shows a pre-existing islet. After THR-123 removal, new insulin+ cells are EGFP-tagged within the β-cell formation at d9. Scale 300 μm (magnified insets: 50 μm). 
c Representative microphotograph of murine pancreatic slices from Ins2-Cre.mTmG mice treated for 7 days with THR-123. Green arrow shows a pre-existing islet. After THR-123 removal, new insulin+ cells are EGFP-tagged within the β-cell formation at d9. Scale 300 μm (magnified insets: 50 μm). d Reporter strategy involving the co-transduction with adenoviral RIP-Cre tracer and lox/LSL-lox reporter strategy. 

### Data and Analysis

**Quantification Panel**: The data for dsRED expression in co-transduced, BMP-7-treated or control slices over 7 days in alloxan-treated mice is presented in the following chart. The chart shows the percentage of slices expressing dsRED in different conditions. For each condition (Control (dsRED), BMP-7 (dsRED), and Alloxan (dsRED)), the percentage of slices expressing dsRED is plotted. The data points are presented as mean ± SD. Each point represents the mean of three technical replicates, while plotted bars/lines are centered at mean. Source data are provided in the Source Data file.
identity of the green cells, when alloxan was added again at this point, all EGFP+ cells disappeared again at 24 h (Fig. 4k).

Based on these results in mice, we sought to replicate this adenoviral-based reporter system using HPSs. Slices were generated from n = 5 donors and treated one day after transduction with 100 ng/ml BMP-7 for 3 days, followed by removal for 3 days (Fig. 5a–d). Since human β-cells are naturally resistant to alloxan, pre-existing islets could not be eliminated as previously done in murine slices. Therefore, co-transduction of the two adenoviral vectors was expected to result in the labeling of resident β-cells, whereas newly formed β-cells would represent a comparatively smaller contribution to the overall EGFP signal. Another prediction of the model was that, in the absence of additional regeneration (i.e., no BMP-7 control), EGFP signal corresponding exclusively to islets would plateau soon after all transduced β-cells turned green. In contrast, BMP-7-mediated formation of new insulin-producing cells would keep gradually increasing in BMP-7-treated slices after controls have plateaued. Confirming these hypotheses, EGFP signal in the BMP-7-treated group continued to expand with time, whereas the islet-labeled area in the control group remained unchanged after day 1 (Fig. 5c, d).

Since the insulin promoter used was not human, we set out to confirm our findings using a synthetic human insulin promoter (SHIP) with heightened specificity for human β-cells (kindly provided by Dr. C. Brunicardi, University of Toledo, OH). Using an adenovirus containing a SHIP-driven EGFP construct, we tested for promoter specificity. As expected, we observed that all SHIP-labeled cells were insulin+ (Fig. 5e and Supplementary Fig. 5a, c, d), but not glucagon+ (Fig. 5e and Supplementary Fig. 5d), somatostatin+ (Supplementary Fig. 5b, d) or detected outside islets (Supplementary Fig. 5e). Next, we co-transduced HPSs (n = 3 donors), treated them with BMP-7 (or vehicle) for 3 days and tracked β-cell formation over time. As before, following the initial labeling of pre-existing islets in both groups (which is delayed vs. that observed with RIP-Cre), we observed a progressive increase of the EGFP-tagged area in the BMP-7-treated group, whereas that of the control group remained largely unchanged. The quantification of this signal showed significant differences in total EGFP-expressing area at days 8–10 (4–6 days after BMP-7 removal), confirming β-cell generation using a human specific promoter system (Fig. 5g, h). Both with RIP and SHIP, waves of yellow-tagged cells (Fig. 5c, h) were more readily detected in BMP-7-treated slices than in controls.

Finally, we repeated the experiment using HPSs from a T2D diabetic donor. While rarer, these samples present us with the unique opportunity to study regeneration in a disease model. As shown in Fig. 5i, j, these samples also exhibited BMP-7-dependent increase of EGFP signal from day 8 onwards vs. controls, suggesting that β-cells can be induced to regenerate in slices from T2D donors.

Discussion

We describe here the long-term culture of HPSs using oxygen-enhancing techniques. These methods resulted in the functional preservation of endocrine and exocrine cells for at least 10 days, a period during which HPSs cultured in control conditions exhibited significant deterioration. The extended preservation of organotypic pancreatic tissue, coupled with adenoviral-mediated lineage tracing, offers a unique opportunity to explore human endocrine regeneration in a culture setting that retains to a significant degree the anatomical integrity of the native organ. While previous reports have lineage-traced human primary pancreatic tissue in vitro32,33, the very act of placing such tissues in culture introduces experimental bias and induces artificial de-differentiation27,32,33. HPSs have circumvented those problems3–7, but their usefulness has been limited to short-term physiological studies due to their rapid degeneration. To our knowledge, no other report on organotypic human pancreatic tissues describes culture for longer than 48 h.

Suboptimal oxygenation is arguably the most critical factor responsible for this loss of viability and function. The thickness of HPSs (120 μm) is at the very limit of passive gas diffusion across living tissue that would ensure survival of cells at its core, provided that an adequate oxygen supply (e.g., a blood vessel) existed at both sides. This is not the case in regular settings, where air has to diffuse through a much longer distance from the surface of the medium to where the tissue is located—and then across it. Our modeling confirms that anoxia occurs rapidly after only 16 h of culture in transwells. Anoxia induces an irreversible loss of respiratory capacity in the pancreas34. This leads not only to cell death but, perhaps more importantly, a metabolic change in the surviving cells, which adapt to lower environmental oxygen by switching to glycolysis. Even though they still represent an improvement over non-organotypic cultures, HPSs in transwells are metabolically aberrant. In contrast, PFC-cultured HPSs maintain low levels of lactate production and glucose
consumption rates after 10 days in culture. Since slices in both groups produce comparable amounts of ATP, but the glucose uptake and consumption are lower in those cultured in PFC, our observations suggest the maintenance of a highly efficient, oxidative phosphorylation-based metabolism. Additionally, PFC-cultured HPSs displayed significantly better viability, cross-sectional area, glucose responsiveness, and overall islet integrity over the studied period. We speculate that this is due to the suboptimal wellbeing of islets in control conditions. Interest-ingly, those improvements were more evident in the endocrine than in the exocrine compartment. With the exception of the cross-sectional area, which was significantly higher in the PFC group, calcium imaging following exposure to acinar cell secre-tagogues and overall TF profiles were largely comparable in both groups at day 10. This may be due to refinements in the medium, which contains two additional protease inhibitors (aprotinin and chymostatin) compared to the formulations reported thus far for HPSs. Still, higher magnification images of the acinar compartment indicate more defined E-cadherin ultrastructure and apical amylase granulation in PFC-cultured slices. Another intriguing observation is that the acute dynamic glucose response profile of HPSs immediately after sectioning was worse than that recorded at day 10, be it on transwells or PFC dishes. This prompted us to test whether resting the samples for 24 h after the stress of slicing would result in their functional recovery, as it happens with islets after isolation. This was indeed the case, as shown in the second round of experiments presented in Fig. 3g.

Our goal in refining the conditions for the long-term survival of HPS was to allow for the real-time detection and quantification of endocrine cell regeneration. Multiple models have been presented in the literature to explain β-cell regeneration, from β-cell duplication and α-to-β cell transdifferentiation to duc-tal progenitor cell differentiation (reviewed in ref. 39). We decided to test the latter (ductal progenitors) owing to our previous discovery of a BMP-7-responsive sub-population of progenitor-like cells in the major ductal tree of the human pancreas. However, our system could as easily be tailored to study other models of regeneration. Proof-of-concept experiments were performed on a transgenic mouse model that was first used to study β-cell neogenesis in adult mice. That report confirmed neogenesis during pancreatic development and in newborn mice, but failed to detect yellow (neogenic β-) cells after post-natal day 5 following a variety of insults to the pancreas. However, we readily detected yellow cells in slices derived from these very same mice after treatment with BMP-7 and THR-123. This apparent contradiction is not such, inasmuch as BMP-induced regeneration was not among the models tested in that study. Notably, we were able to replicate this in slices from non-transgenic mice, and subsequently from humans, by means of adenoviral co-transduction of a red-green reporter and an insulin lineage tracer. As predicted, the combination of long-term slice culture and virally-mediated lineage tracing allowed us not only to detect, but to quantify BMP-7-induced regeneration events longitudinally. The relatively low frequency of co-transduction was not an obstacle to observe transitional yellow and subsequently permanent green events in HPSs, both with the rat insulin promoter (RIP) and with a human synthetic insulin promoter (SHIP). Of note, owing to its molecular design, the SHIP promoter could be potentially activated in PDX1 cells that do not express insulin. However, we did not observe such occurrence upon transduction of human pancreatic slices with SHIP-Cre (Fig. 5e and Supplementary Fig. 5). Although the relative percentage of green events was lower when using the latter (suggesting a higher specificity of this promoter), the differences were not statistically significant.

The increase in EGFP population is statistically significant in the BMP-7 group vs. control. While the increase in the “yellow” population (i.e., cells that are transitently both EGFP- and dsRED-positive, reflecting new onset of insulin expression) is not statistically significant, it is important to stress that, while green signal is cumulative, yellow is not. Unlike the green signal, the yellow that we detect at the time of imaging may or may not be re-presentative of what happens over a 24 h period. Therefore, determin-ing true statistical significance based on the arbitrary temporal measurement of cells that are yellow only for a short period may not be possible.

An observation that stands out from the murine slice experiments is that new green cells are also detected in controls (non-BMP-7-treated), although at a significantly lower level. Both groups were treated with a β-cell toxin (alloxan), which in itself is a stimulus for regeneration. This effect is likely aided by the high concentration of BMPs present in the serum used to supplement the murine (but not the human) culture medium. In HPSs, we did not detect any increase of EGFP signal in controls as the initial tagging of pre-existing β-cells. In contrast, BMP-7-treated slices displayed significantly stronger waves of red-green colocalization, suggesting additional formation of β-cells after the initial labeling of the resident ones.

Importantly, our analyses are merely suggestive of β-cell neo-gensis. The unequivocal demonstration of this phenomenon would require additional lineage-tracing as well as endocrine cell turnover determinations. Future work on such approaches will shed additional light on these observations. In addition, owing to the use of longitudinal slice-wide, low-magnification imaging (which is necessary to address the uncertainty about the location in the slice where new insulin+ cells may appear), transitional events cannot be resolved at the single cell level. However, while higher-resolution alternatives are developed, our analyses are still valid to quantify overall fluorescence changes at the tissue level. It must also be stressed that the in-depth mechanistic analysis of the reported observations (e.g., the functional analysis of newly formed insulin-producing cells) is beyond the scope of this report, whose main focus is the description of a method for the long-term, real-time monitoring of regeneration in live pancreatic cultures that preserve the tissue cytoarchitecture and cell-to-cell interactions of the native organ. This approach fills a void in the field and will enable investigators to study regeneration in the human pancreas with an unparalleled degree of resolution. If applied to samples from T1D and T2D donors, long-term cultured HPSs may offer new insights on the pathology—and potential treatment—of diabetes.

Methods
COMSOL modeling–finite element modeling of oxygen distribution. 2D diffusion reaction modeling of oxygen distribution within the pancreatic slices was performed using COMSOL v5.3 (Comsol Inc, Burlington, MA). A parametric sweep of oxygen consumption rates (1.0–5.0 × 10−3 mol/m² s−1) unique to pancreatic tissue was performed. The only conditions that differed between the two culture models were the basal surface boundary condition with continuity utilized for the transwell suspended in culture medium above the impermeable plastic surface, and a concentration boundary at the basal surface of the oxygen sandwich representative of the interface of the surface with the surrounding bulk oxygen. All diffusivity was represented as diffusive permeability, the product of effective diffusivity and the oxygen solubility in a given phase, in order to eliminate the need for partition coefficients and to express the oxygen distribution in terms of partial pressure (mmHg). Tissue anoxia was represented by a pO2 of <0.1 mmHg per literature convention in regards to pancreatic islets and acinar tissue. Anoxic tissue volume was calculated as the ratio of pancreatic slice volume with a pressure (mmHg). Tissue anoxia was represented by a pO2 of <0.1 mmHg per
measurement of metabolic activity. Media analyses were measured on a Beckman Coulter Vii-CELL MetaFLEX to determine glucose consumption, lactate production and osmolarities of the culture sample. At the end of each time point, a media blank was performed to normalize all pancreatic slice samples. In culture samples, evaporation is a confounding factor, so all values were corrected using the relative osmolarity of the culture sample relative to the media blank expressed in the following equations for glucose consumption and lactate production:

\[
GCR = \frac{Osm_{blank} - (Osm_{culture} \times \frac{Time}{\Delta Time})}{\Delta Time}
\]

\[
LPR = \frac{Lactate_{culture} - Lactate_{blank}}{\Delta Time}
\]

All values for GCR were expressed as (μg consumed/produced per hour)/μg DNA, while values for LPR were expressed as (μg produced/consumed per hour)/μg DNA.

Glycogen uptake was measured as follows: slices were pre-incubated in glucose-free BrainPhys medium (StemCell Technologies, Vancouver, BC, Cat# 05790) containing 2% B27 supplement minus-insulin (Invitrogen, Carlsbad, CA; Cat# A1895601) for 40 min. After washing 3X in PBS, slices from each group (PFC or TW) were incubated for 10 min in regular BrainPhys medium (see composition below) supplemented with 100 µM of 2-NBDG (StemCell Technologies, Vancouver, BC, Cat# 05790). After incubation, slices were immediately washed 1X in PBS, briefly incubated with propidium iodide (PI), washed again 3X; briefly incubated with DAPI, washed again 3X, and immediately imaged using a confocal microscope.

DNase quantification of slice tissue. Pancreatic slices were digested using T-PER Tissue Protein extraction reagent (Invitrogen, Carlsbad, CA; Cat# 78510). Supernatant was then used to calculate total DNA quantity using a Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, CA; Cat# P7589) according to the manufacturer’s recommendations.

Transgenic mouse models. All animal experiments were conducted under the supervision and oversight of the University of Miami Institutional Animal Care and Use Committee (IACUC) and Division of Veterinary Resources (DVR) at the University of Miami. CD1-IGS mice (5–6 weeks old; Charles River, Wilmington, MA, Cat# 022) were utilized for pancreatic slice tissue to obtain control slices for adenoaviral transduction experiments. In order to create the IN52-Cre/mTom red fluorescent reporter, we bred B6.Cg-Tg(In2-Cre)25Mmpf/ (INS2-Cre; Jackson Labs, Bar Harbor, ME, Cat# 003573) with B6.Gt(Rosa26Sorcre/ACTB-tdTomato,EGFP)J/Mtmg; Jackson Labs, Bar Harbor, ME, Cat# 007676). In the resulting mouse, all insulin-producing cells (ventro-medial hypothalamus, pancreatic β- and δ-cells, data not shown), express fluorescent EGFP, while all non-insulin-producing cells express fluorescent tdTomato. We used only F1 generation mice for β-cell formation experiments. Stability of the mTmG reporter was confirmed and tracking β-cell generation in pancreatic slices. All mice were housed in specific pathogen-free (SPF) conditions at the University of Miami’s vivarium and were maintained on a 12 h light/dark cycle. Slices were transplanted into a humidified cell incubator at 37 °C for at least 45–90 min prior to usage. Once transduced, the dishes were carefully washed with ice-cold DBS (Sigma Aldrich, St. Louis, MO, Cat# D8537) 3X times. Medium and culture conditions for murine slices: washed murine slices were cultured using murine slice culture medium at pre-coated AirHive dishes or Corning Transwell® polyester membrane cell culture inserts (Corning, NY; Cat #CLS3450-24EA) as controls. The formulation includes custom-made Waymouth’s MB 752/1 medium (with β-glucuronidase; without glucose; Biological Industries, Cromwell, CT, Cat# 06-1110-A1) containing 11 mM D-Glucose (Sigma Aldrich, St. Louis, MO, Cat# G5872), 1% heat-inactivated FBS (Invitrogen, Carlsbad, CA, Cat# 16104063), 100 µg/ml trypsin inhibitor from Glycine max (Sigma Aldrich, St. Louis, MO, Cat# T65522), 10 µg/ml aprotinin (Sigma Aldrich, St. Louis, MO, Cat# A1060), 10 µg/ml chymostatin (solubilized initially in DMSO; Sigma Aldrich, St. Louis, MO, Cat# A1895601), 1% penicillin-streptomycin-amphotericin B solution (Sigma Aldrich, St. Louis, MO, Cat# A5955), 1% Glutamax supplement (Thermo Fisher/Life Technologies, Waltham, MA, Cat# 35060-061), 1X B27-minus Insulin (Invitrogen, Carlsbad, CA; Cat# A10483-01) were added to the culture medium. Dishes were coated with 250 µl/unit, making sure that the entire surface was covered by gently rotating the dish from side to side, and placed in a humidified cell incubator at 37 °C for 45–90 min prior to usage. Once coated, the dishes were carefully washed with ice-cold DBS (Sigma Aldrich, St. Louis, MO, Cat# D8537) 3X times.

Adenoviral vectors. Recombinant adenoaviruses were constructed using the strategy of adenoaviral with an E1/E3 deletion. A co-cultivated Cre variant was used. The Adv-RIP-Cre was created as in23. The SHIP was a gift from Dr. C. Brunacardi (U. of Toledo, OH). The SHIP construct was cloned upstream of an adenovirus packaging plasmid to create the Adv-(CMV)-LoxP-SHIP-Cre adenovirus. The reporter control was a gift from Dr. P. Ravassard, Hôpital Pitié-Salpétrière-Paris, France, and was loaded onto an adenoviral packaging plasmid to create the Adv-(CMV)-LoxP-dsRED-STOP-loxP-EGFP adenoviral reporter. All adenoviral construction was performed at Vector Bioslabs, Malvern, PA. Slices were transduced with a MOI of 50 for the reporter and a MOI of 10 for SHIP-containing viruses.

Viability assessment. Pancreatic slices were incubated with calcine-AM to indicate intracellular calcium activity (live cells) and ethidium homodimer-1 (dead cells). Addition of both of these reagents was done according to the manufacturer’s recommendations as part of the Live/Dead viability/cytotoxicity kit for mammalian cells (Invitrogen, Carlsbad, CA; Cat# L5224). Once stained, slices were washed 3X times with DPBS (Sigma Aldrich, St. Louis, MO, Cat# D8537) and fixed with 3.7% paraformaldehyde. Fixed slices were counterstained with DAPI (Thermo Fisher/Life Technologies, Waltham, MA, Cat# D1306) in DPBS (Sigma Aldrich, St. Louis, MO, Cat# D8537).

Dynamic glucose-stimulated insulin/glucagon release studies. Dynamic glucose-stimulated insulin/glucagon release (perfusion) assays were performed using pancreatic slices (3–4 slices per perfusion chamber in duplicates). Slices were perfused within a perfusion chamber (Warner Instruments, Holliston, MA, Cat# 64-0378). The chamber rests on top of a chamber platform (Warner Instruments, Holliston, MA, Cat# 64-0281). Perfusion was done in Krebs buffer containing 125 mM NaCl, 5.9 mM KCl, 1.28 mM CaCl2, 1.2 mM MgCl2, 25 mM HEPES, and 0.1% bovine serum albumin, pH 7.4 at 37 °C, using a PER3S machine with a fluid recirculation external peristaltic pump. When loading slices into the chamber, slices were incubated in Krebs buffer containing 3 mM glucose on a shaker set at 120 rpm at 37 °C, for 60–90 min. Slices were then loaded
in the chamber using a fine brush of 2/0 size (Electron Microscopy Sciences, Hatfield, PA, Cat# 66100-20) and connected to the perfusion system. Samples were challenged with either low or high glucose (G1 = 1 mM; G2 = 3 mM; G167 = 16.7 mM) or potassium chloride (KCl = 30 mM) at a flow rate of 100 µL/min. After 90 min of flushing in G3 solution, slices were stimulated with the following sequence: 16 min G3, 20 min G167, 30 min G1, 5 min G3 + KCl, and 15 min G3. Samples were collected every 60 seconds into a 96-well plate kept at 4 °C, while the perfusion chamber and perfusion solutions were maintained at 37 °C. After the perfusion, slices of one chamber were fixed in 4% paraformaldehyde for 30 min at room temperature and stored in PBS at 4 °C for insulin staining and subsequent β-cell quantification. Slices from the second chamber were placed in 500 µl of acid ethanol and stored at −20 °C. Peroxidase staining and stored at −20 °C. Immunofluorescence analysis. Tissue slices were washed 2× for 5 min in 1X phosphate buffered saline (PBS), pH 7.4 (Sigma Aldrich, St. Louis, MO, USA Cat# P3813), and fixed in 3.7% paraformaldehyde solution overnight. Slices were then washed 10× for 5 minutes in 1X phosphate buffered saline (PBS), pH 7.4 (Sigma Aldrich, St. Louis, MO, USA Cat# P3813). Permeabilization was done for 30 minutes using 0.3% Triton (Sigma Aldrich, St. Louis, MO, USA Cat# T9284-500ml) in 1X PBS, pH 7.4 (Sigma Aldrich, St. Louis, MO, USA Cat# P3813). After this, slices were blocked in blocking buffer (DH2O, 5% normal goat serum, 1% bovine serum albumin, 0.5% Triton X-100, 1%ivic) of the slices to allow for quantification within the sensitivity range.

Dynamic glucose-stimulated alpha-Amylase release studies. Pancreatic amylase secretion was evaluated using 3X human pancreatic slices placed in a perifusion chamber. Perifusion was similar to that described for glucose secretion, with some modifications. Krebs buffer was supplemented with 10 µg/ml aprotinin (Sigma Aldrich, St. Louis, MO, USA Cat# A6106), 3 mM β-glucose (Sigma Aldrich, St. Louis, MO, USA Cat# G6664) and 1X penicillin-streptomycin-amphotericin B solution. Slices were transferred between either 3 mM Glucose (3 min) or 100 µM Carbachol (Sigma Aldrich, St. Louis, MO, USA Cat# C2901), or 3 mM Glucose + 100 µM Carbachol (Sigma Aldrich, St. Louis, MO, USA Cat# C212385-M). Perifusates were collected after every 120 s in a 96-well plate kept on ice at all times. Amylase quantification was calculated using a calorimetric assay for amylase activity, based on the manufacturer’s recommendations (BioVision, Milpitas, CA, Cat# K711-100).

Quantification of cytosolic Ca²⁺ levels. Confocal images (pinhole = airy 1) of randomly selected islets were acquired on a Leica SP5 confocal laser-scanning microscope with 40X magnification (NA = 0.8). Pancreatic tissue slices were reconstructed in Z-stacks of 15–30 confocal images (step size = 2.5–5.0 µm) and analyzed using ImageJ. [Ca²⁺], responses were quantified as the areas under the curve of individual traces of Fluo-4 fluorescence intensity during the application of the stimulus. Changes in intracellular Ca²⁺ levels were measured using ImageJ. Changes in fluorescence intensity are expressed as percentage changes over baseline (ΔF/F). We measured changes in total cytosolic Ca²⁺ levels by computing the area under the curve above baseline using Prism software (Prism 7, GraphPad software, La Jolla, CA). Areas under the curve were determined before, during, and after each stimulus for the same time period and compared with statistical tests. For quantification of [Ca²⁺], responses, we calculated the areas under the curve of the fluorescence intensity traces of Fluo-4. Our criteria for acceptance of [Ca²⁺], responses, for analyses were that: (1) responses could be elicited ≥2× the same stimulus; and (2) the peak signal was ≥2× the baseline fluctuation. For quantification of β-cell responses, we selected cells that responded to increases in [Ca²⁺], during KC1 and high glucose (16 mM) stimulation, in order to exclude dendritic a-cells from our quantification.

Immunofluorescence analysis. Tissue slices were washed 2× for 5 min in 1X phosphate buffered saline (PBS), pH 7.4 (Sigma Aldrich, St. Louis, MO, USA Cat# P3813), and fixed in 3.7% paraformaldehyde solution overnight. Slices were then washed 10× for 5 minutes in 1X phosphate buffered saline (PBS), pH 7.4 (Sigma Aldrich, St. Louis, MO, USA Cat# P3813). Permeabilization was done for 30 minutes using 0.3% Triton (Sigma Aldrich, St. Louis, MO, USA Cat# T9284-500ml) in 1X PBS, pH 7.4 (Sigma Aldrich, St. Louis, MO, USA Cat# P3813). After this, slices were blocked in blocking buffer (DH2O, 5% normal goat serum, 1% bovine serum albumin, 0.5% Triton X-100, 1%ivic) of the slices to allow for quantification within the sensitivity range.

An ApoTeo Axiovert 200M (Zeiss) fluorescent microscope, with the slices positioned in similar orientations as the preceding day while atop the AirHive. End-point imaging was performed using a Leica MP-ND4/SP5/FS3/FILM multiphoton/confocal upright F-techniques microscope MP/SP5 having a laser set at 30% intensity, pinhole set at 1, airy set at 1, with 10X and 20X magnification. Z-stack depth ranged between 120 and 130 µm depending on the slice thickness and condition, while the number of images in the Z-stack ranged between 15 and 25 with a slice spacing ranging between 2.5 and 4 µm. To prevent bias, for eGFP/3dTomato and viability studies we utilized ImageJ/FIJI to perform quantitative analysis of cell populations, using total area calculations for fluorescent area and correlating to brightfield slice area. In additional quantitation of images, DAPI-positive were counted to determine the total number of cells, against which all the other markers were quantified.

HIFix determination. ELISA (MyBioSource #MBS2885065) was performed to assess the levels of HNF6a comparing slices cultured in transwells and PFC. The results were normalized to total tissue protein content using a Pierce™ BCA assay (Thermo Scientific #23227).

Quantitative real-time RT-PCR. qRT-PCR was performed with the Applied Biosystems/Thermo Fisher Scientific (Waltham, MA) TaqMan® assays listed in the Supplementary Data 1 (table of key resources).

Statistical analysis. Statistical analyses and graphing were performed using GraphPad Prism v8 (GraphPad software, La Jolla, CA). Following the Shapiro–Wilk normality test, statistical differences between groups were calculated by two-tailed paired t test or Wilcoxon signed-rank test, with 95% confidence intervals (p < 0.05; p < 0.01; p < 0.001). Results are expressed as mean ± SD. Statistical comparisons for cytosolic Ca²⁺ levels were performed using Student’s t test or one-way ANOVA followed by multiple-comparison procedures with the Tukey or Dunnett’s tests. Data are shown as mean ± SEM.

Key resources. A table of key resources used in this manuscript is provided as Supplementary Data 1.

Data availability. All relevant data are available from the authors. Source data underlying Figs. 1e–g: 3d-g; 3d-l; 4h–j; k; and 5b–d; g–h are provided as a Source data file.

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Competing interests
The University of Miami and Drs. Dominguez-Bendala, Fraker and Ricordi hold, but do not receive royalties for intellectual property used in this study. They are also equity owners in Ophysio, Inc., licensee of the intellectual property. The remaining authors declare no conflicts of interest.

Additional information
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