Artificial three-dimensional niches deconstruct pancreas development *in vitro*

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SUMMARY

In the context of a cellular therapy for diabetes, methods for pancreatic progenitor expansion and subsequent differentiation into insulin-producing beta cells would be extremely valuable. Here we establish three-dimensional culture conditions in Matrigel that enable the efficient expansion of dissociated mouse embryonic pancreatic progenitors. By manipulating the medium composition we generate either hollow spheres, which are mainly composed of pancreatic progenitors, or complex organoids that spontaneously undergo pancreatic morphogenesis and differentiation. The *in vitro* maintenance and expansion of pancreatic progenitors require active Notch and FGF signaling, thus recapitulating *in vivo* niche signaling interactions. Our experiments reveal new aspects of pancreas development, such as a community effect by which small groups of cells better maintain progenitor properties and expand more efficiently than isolated cells, as well as the requirement for three-dimensionality. Finally, growth conditions in chemically defined biomaterials pave the way for testing the biophysical and biochemical properties of the niche that sustains pancreatic progenitors.

KEY WORDS: Bioengineering, Branching, Polarity, Diabetes

INTRODUCTION

A cellular therapy for diabetes is currently limited by the supply of beta cells. Robust protocols to produce endoderm from embryonic stem cells or induced pluripotent stem cells are available and production of pancreatic progenitors after endoderm induction is becoming possible (Kroon et al., 2008; Ameri et al., 2010; Cheng et al., 2012). Yet, two major challenges remain as obstacles to the mass production of beta cells *in vitro*: (1) the induced population of pancreatic progenitors must be expanded; and (2) the efficient and reproducible differentiation of progenitor cells into glucose-responsive, insulin-producing beta cells must be achieved.

Before embryonic day (E) 11.5 in mouse, the early pancreas consists essentially of multipotent pancreatic progenitors that can give rise to all pancreatic cell types (Gu et al., 2002; Seymour et al., 2007). These cells express *Sox9*, *Pdx1*, *Hnf1b* and *Ptf1a*. By E14.5 they have given rise to two progenitor populations, which are restricted in their developmental potential. *Pdx1*^{+}/*Ptf1a*^{+} cells located at the terminal end buds of the epithelial tree are irreversibly committed to an acinar fate. A subset of these cells expresses low levels of SOX9 and lineage tracing has shown that SOX9^{+} cells can contribute to acini at E14.5 (Furuyama et al., 2011; Kopp et al., 2011). *Hnf1b*^{+} cells, also expressing *Sox9* and *Pdx1*, are located in the central ducts and are bipotent progenitors that can give rise to both ductal and endocrine cells (Kopinke and Murtaugh, 2010; Zhou et al., 2007; Solar et al., 2009). *Neurog3*^{+} endocrine progenitors originate from multipotent and bipotent pancreatic

progenitors at all stages and can give rise to endocrine cells (Gu et al., 2002; Seymour et al., 2007).

In vitro cultures of pancreas explants have been useful for the imaging of processes that occur during pancreas development and for drug/pathway testing (Miralles et al., 1998; Percival and Slack, 1999). For many organs, the ability to culture dispersed primary cells has provided invaluable access to cell lineage relationships and the intrinsic properties of isolated cell types (Hope and Bhatia, 2011). In pioneering work, Sugiyama et al. (Sugiyama et al., 2007) isolated different populations from the embryonic pancreas, maintained *Neurog3*^{+} endocrine progenitors in culture for 5 days and observed their differentiation into endocrine cells in low-density cultures on feeder layers. CD133^{+} CD49f^{high} *Neurog3*^{−} progenitors could also survive *in vitro* for 3 days on feeders.

Expanding multipotent pancreatic progenitors have not been found *in vivo* in the adult uninjured pancreas and their appearance during injury is subject to controversy (Xu et al., 2008; Kopp et al., 2011). Nevertheless, it is possible that an expanding multipotent population exists in the adult. Indeed, multicellular spheres ('pancreatospheres') can be generated at low efficiency (1 in 4×10^4) from isolated adult pancreatic ducts or islet cells. However, these cells gave rise to both pancreatic cells and neurons, therefore also producing cell types that are normally not derived from pancreatic progenitors (Seaberg et al., 2004; Smukler et al., 2011). Recent experiments have also led to the isolation of a rare (1%) SOX9^{+} adult ductal cell population that could also form hollow spheres and give rise to endocrine cells (Jin et al., 2013).

Recently the expansion of miniorgans or hollow spheres using three-dimensional (3D) culture conditions has been reported from the intestine (Ootani et al., 2009; Sato et al., 2009), stomach (Barker et al., 2010), liver (Huch et al., 2013), mammary gland (Dontu et al., 2003), prostate (Lukacs et al., 2010) and trachea (Rock et al., 2009). Here, we identify 3D *in vitro* culture conditions in which single or small groups of embryonic pancreatic progenitors vastly expand, branch similarly to the pancreas and differentiate. We test how faithfully they recapitulate pancreas development and use them to discover new aspects of pancreas organogenesis.

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MATERIALS AND METHODS

Animals

E0.5 was defined as noon of the day when the vaginal plug was detected in the mother. We used the following genetically modified mouse lines, backcrossed to the outbred CD1 strain (Harlan): *Neurog3* knockout (Gradwohl et al., 2000); *Pdx1-Ngn3-ER^TM-ires-nGFP* (Johansson et al., 2007); *Sox2-Cre* (Hayashi and McMahon, 2002); *R26R-YFP* (Srinivas et al., 2001); *R26R-lacZ* (Komada and Soriano, 1999); *Ngn3(EYFP)* (Mellitzer et al., 2004); *Gt(ROSA)26Sor^tm1(Notch1)Dam* (Murtaugh et al., 2003); and *Pdx1-Cre* (Hingorani et al., 2003). The Swiss and Danish Veterinary Offices approved the animal experiments performed in the respective countries.

Preparation of pancreas progenitor suspension and Matrigel culture

Embryonic dorsal pancreata were harvested from E10.5 embryos unless otherwise indicated. The mesenchyme was removed with tungsten needles and the remaining epithelial bud further cleaned from mesenchymal contaminants with dispase (1.25 mg/ml; Invitrogen). Epithelial cells were incubated in 3-10 μl 0.05% trypsin (Gibco) at 37°C for 5 minutes in 60-well Minitrays (Nunc) and inactivated in 3-10 μl Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal calf serum (FCS). Cells were dissociated to a single-cell suspension using a thin pulled capillary. The cell suspension obtained from pooled pancreata was mixed with growth factor-depleted Matrigel (BD Biosciences) at a 1:3 ratio and aliquoted into a 96-well plate at 8 μl/well with 5-8 cells/μl, i.e. ~40-50 cells/well. To solidify the Matrigel the plate was incubated for 5 minutes at 37°C, and the cells then grown in 70 μl medium/well. Medium was replaced every 3-4 days and the growing pancreatic colonies were monitored daily.

The organogenesis medium composition was based on the previously reported *in vivo* requirements of pancreas progenitors and was inspired by media used for *in vitro* culture of digestive tract-derived progenitors or stem cells (Miettinen et al., 2000; Bhushan et al., 2001; Cras-Méneur et al., 2001; Sugiyama et al., 2007; Rock et al., 2009; Sato et al., 2009). The composition is as follows: DMEM/Nutrient Mixture F12 (DMEM/F12; Gibco); 1% penicillin-streptomycin; 10% KnockOut Serum Replacement (KSR; Gibco); 0.1 mM 2-mercaptoethanol; 16 nM phorbol myristate acetate (PMA; Calbiochem); 10 μM Y-27632 (ROCK inhibitor; Sigma); 25 ng/ml EGF (Sigma); 500 ng/ml mouse R-spondin 1 (R&D Systems); 100 ng/ml FGF10 (R&D Systems); 25 ng/ml FGF1 (R&D Systems); 2 U/ml heparin (Liquemin; Roche). Organoids formed efficiently from the CD1 strain and from the *Ngn3(EYFP/+)*, *Sox2-Cre x R26R-lacZ*, *Sox2-Cre x R26R-YFP* and *Pdx1-Ngn3-ER^TM-ires-nGFP* (in the absence of the tamoxifen that would induce NEUROG3 function) strains. Tests with additional 10% FCS did not improve the system and, conversely, led to reduced branching. Where indicated, mouse recombinant noggin (a gift from J. Huelsken's group, ISREC SV EPFL, Lausanne) was added to the medium at a final concentration of 50 ng/ml. DAPT {(*N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester} (Sigma) stocks at 12.5 mM in DMSO were used at a final concentration of 5 μM or 10 μM. A 1:250 dilution of DMSO was added to all final medium compositions. SU5402 (Calbiochem) stocks at 10 mM in DMSO were diluted to 10-40 μM. The medium designed to maintain progenitors in spheres comprises: DMEM/F12 (Gibco); 1% penicillin-streptomycin; 1× B27 culture supplement (GIBCO); 64 ng/ml FGF2 (R&D Systems); 10 μM Y-27632 (ROCK inhibitor; Sigma). Spheres formed efficiently from the following strains: CD1, *Sox2-Cre x R26R-lacZ*, *Sox2-Cre x R26R-YFP* and *Pdx1-Ngn3-ER^TM-ires-nGFP* (without tamoxifen).

3D culture in hydrogels

Initial synthetic hydrogel experiments were performed with commercially available poly(ethylene glycol) (PEG) precursors (QGel, Cat. #1001). The lowest achievable stiffness with these gels was Young's modulus (G') = 1 kPa. In order to obtain hydrogels of lower stiffness, subsequent experiments were carried out with custom synthesized enzymatically cross-linked PEG hydrogels. PEG vinylsulfone (PEG-VS) was produced and characterized as described (Lutolf et al., 2003). PEG-VS was functionalized with peptide substrates of the FXIIIa enzyme via Michael-type addition. We used a glutamine-containing peptide (NQEQVSPL-ERCG-NH$_2$) and lysine-containing peptides with MMP-sensitive sequences: AcFKGG-GPQGIWGQ-ERCG-NH$_2$. Functionalization and characterization of these precursors was performed as previously described (Ehrbar et al., 2007). Precursor solutions to provide hydrogels with a final dry mass content of 1.5% (corresponding to a G' of ~250 Pa) were prepared by stoichiometrically balanced ([Lys]/[Gln]=1) solutions of the glutamine and lysine PEGs in Tris-buffered saline (TBS, 50 mM, pH 7.6) containing 50 mM calcium chloride. The cross-linking reaction was initiated by 10 U/ml thrombin-activated factor XIIIa and vigorous mixing. The matrices were then incubated for 30 minutes at 37°C. Laminin (from Engelbreth-Holm-Swarm mouse tumor; BD Biosciences) was used at a final concentration of 0.5 mg/ml. Large extracellular matrix proteins such as laminin function as natural substrates for FXIIIa and tether to the hydrogel network without further conjugation. This was demonstrated using a fluorescent binding assay in which proteins were shown to react with fluorescent FXIIIa substrates (Q-peptide-Alexa 647 or Lys-Tamra) in the presence of FXIIIa.

2D culture

We functionalized 96-well plates with either pig skin gelatin (0.1%; Sigma) or laminin (0.16 mg/ml; BD Biosciences). After 30 minutes of incubation in the wells, the protein solution was removed and E10.5 isolated pancreatic progenitors were seeded at 5-10 cells/μl. To test the 'on Matrigel' condition, Ibidi chambers (ref. 81506) that minimize meniscus formation were used. 10 μl chilled Matrigel was deposited in the lower chamber and, once solidified, 50 μl resuspended E10.5 pancreatic progenitors were seeded at 5-10 cells/μl. Medium was changed at day 4.

Transplantation assay in explants

Pancreatic progenitors were collected from *Sox2-Cre x R26R-YFP^+* E10.5 embryos and processed as described above. They were cultured for 6 days and then harvested to be grafted into recipient pancreatic explants. Recipients were dorsal pancreata from E12.5-13.5 wild-type CD1 embryos, freshly dissected and seeded in culture on the day of the graft. Pancreatic organoids were grafted after rough dissociation in 0.05% trypsin and inactivation in DMEM + 10% FCS, or grafted directly after mechanical dissociation. The graft was injected into the recipient with a mouth pipette, close to the epithelium. The experiment was repeated twice with a total of ~30 pancreatic organoids efficiently grafted into 37 recipient explants. The explants were cultured on Millicell 0.4 μm culture inserts (Millipore) at the air-liquid interface in 4-well plates with M199 + 10% FCS (Invitrogen), 1% penicillin-streptomycin, 1% Fungizone (Invitrogen). The culture was stopped 3, 4 or 6 days after the graft. The explants were fixed for 15 minutes in 4% paraformaldehyde (PFA) and analyzed by immunohistochemistry.

Immunohistochemistry

The tissue was fixed for 15 minutes in 4% PFA and embedded as previously described (Johansson et al., 2007). Consecutive cryosections (7 μm) were collected in series of four to six slides. Frozen sections were dried at room temperature and washed in phosphate-buffered saline (PBS). Cells were permeabilized in 0.25% Triton X-100 for 10 minutes at room temperature. The blocking step was either in 10% FCS and 0.1% Triton X-100 in TBS or in 1% BSA in TBS with a 45-minute incubation at room temperature. Primary antibodies (supplementary material Table S1) were diluted in the blocking solution and incubated with the samples overnight at 4°C. After washing off the unbound primary antibody, secondary antibodies were applied for 1 hour at room temperature and in a sequential manner when multiple antibodies were used. Alexa Fluor (A568, A488; Pacific Blue- or A568-conjugated streptavidin; Molecular Probes-Invitrogen) and biotinylated (Jackson ImmunoResearch Laboratories) secondary antibodies were used for multicolor detection. To stain nuclei DAPI (4′,6′-diamidino-2-phenylindole; 50 ng/μl; Sigma) or DRAQ5 (1:500; Biostatus) was applied.

For the proliferation assay based on EdU incorporation, we administered 10 μM EdU *in vitro* 2 hours before fixation and detected the EdU using the Click-iT EdU Kit (Invitrogen) following the manufacturer's protocol.

Microscopy

Histology images from fluorescence microscopy were taken with a Zeiss Axioplan 2, a Leica DM5500 upright microscope or a Zeiss LSM700 confocal microscope. Cultured cells on 2D substrates or in Matrigel were imaged with either a Leica DMI4000 inverted fluorescence microscope equipped with a DFC 340FX camera or an Eclipse Ti microscope (Nikon) equipped with a Retiga 2000R camera (QImaging). All images were then reframed to match figure size and adjusted homogeneously using the Adobe Photoshop CS3 brightness and contrast function. Images of organoids were captured at the depth with the widest surface using a long-distance 10× objective and area measurements were performed using ImageJ (Fiji).

Time-lapse imaging

Pancreatic progenitors from $Pdx1$-$Ngn3$-ER^{TM}-$ires$-$nGFP^+$ E10.5 embryos were resuspended in Matrigel as described above. Small droplets (3 µl) deposited into 4-well plates filled with 5 ml medium (Nunclon Delta-treated surface, Nunc-Thermo Fisher Scientific) were found to be optimal for imaging. Time-lapse imaging in a humidified, heated, CO_2-controlled chamber was started 3 hours after seeding the cells using a cell^R imaging station (Olympus), under a 10× objective. One picture was taken per hour over a recording duration of 60-63 hours, monitoring 120 positions in parallel. For every position we acquired a differential interference contrast (DIC) image and the GFP signal, reporting $Pdx1$ expression. Pictures of the same positions were acquired once a day after the end of the time-lapse experiment to monitor the evolution from small clusters to organoids. Seven days after the initial seeding, the organoids were fixed in 4% PFA for 15 minutes. The experiment was repeated twice for each culture medium (organoid and sphere). A total of 158 single cells, 92 doublets and 59 small clusters were monitored in the organoid medium and 104 single cells, 54 doublets and 65 small clusters were monitored in the sphere medium.

For data analysis, the initial number of cells at a location was determined, as well as the maintenance of $Pdx1$ expression (at least one $Pdx1^+$ cell), the division of $Pdx1$/nGFP$^+$ cells, death (from phase) and other events (fusion of different seeds, loss of focus, and migration out of field).

Similar methods were used to observe 115 progenitor clusters from $Ngn3(EYFP)$ E10.5 embryos (Mellitzer et al., 2004), marking endocrine progenitors. However, organogenesis medium without FGF1 was used to boost endocrine cell production.

Quantitative PCR

Total RNA was extracted as described (Gouzi et al., 2011) and cDNA was synthesized using the Superscript III reverse transcriptase system (Invitrogen). Quantification of the transcript was performed using 2× SYBR Green Master Mix (Applied Biosystems) with the Step One Plus system (Applied Biosystems). Samples were heated to 50°C for 2 minutes followed by 95°C for 2 minutes, then underwent 50 cycles of PCR amplification (denaturation at 95°C for 15 seconds, primer annealing at 60°C for 25 seconds and elongation at 73°C for 30 seconds) and finally underwent melting curve analysis before cooling to 4°C. mRNA levels of $Hprt$ were used as an internal reference to normalize transcript levels, and changes in mRNA levels after Notch inhibition were determined for $Pdx1$, $Sox9$ and $Hes1$ using the primers indicated in supplementary material Table S2. All gene-specific quantifications were calculated as ΔC_t [target C_t – housekeeping ($Hprt$) C_t] relative to untreated cell experiment control to give a final ΔC_t (test)/ΔC_t (E10.5). Results from triplicate wells in two experiments were averaged and normalized relative to the expression of the E10.5 pancreas. Data are expressed as mean ± s.d. and statistical analysis was performed using an unpaired Student's t-test, comparing the C_t values of each individual tissue of interest. Differences were considered significant at $P<0.05$.

Single-cell qRT-PCR

Single cells were isolated from E10.5 CD1 embryos or 6-day organoid culture and dispersed as described above. Individual cells were handpicked with pulled capillary pipettes under microscopic inspection, and placed into 5 µl Cell Direct 2× reaction mix (Invitrogen) on a glass coverslip and stored at −80°C until further processing. After storage, 4 µl of a solution containing a mixture of SuperScript III reverse transcriptase Platinum, Primer Mix

(500 nM) and DNase/RNase-free distilled water was added. The samples were incubated at 50°C for 15 minutes, 95°C for 2 minutes, and then 22 cycles of 95°C for 15 seconds and 60°C for 4 minutes. Unincorporated primers were removed by adding 3.6 µl exonuclease I (E. coli) at a final concentration of 4 U/µl at 38°C for 30 minutes, followed by inactivation at 80°C for 15 minutes. The final reverse transcriptase-specific target amplification was diluted 1:5 by adding 32.4 µl TE buffer (Teknova). After pre-amplification, the samples and primers (Deltagene, see supplementary material Table S2) were loaded in the 96.96 Dynamic Array (Fluidigm) and primed using the MX IFC controller and then the BioMark Array System (Fluidigm) as recommended by the manufacturer (70°C for 6 minutes for thermal mixing, hot start for 1 minute at 95°C, and 30 cycles of 96°C for 5 seconds and 60°C for 20 seconds). Two to four technical replicates per plate were used to ensure reproducible technical handling. Initial data analysis was performed with the Fluidigm real-time PCR analysis software (v. 3.1.3) with linear derivative baseline correction and a quality correction set to 0.65 in order to eliminate amplification curves with an atypical shape. Data points with aberrant melting curves were eliminated from further analysis. All C_t values exceeding 25 were set to 25, indicating non-expression, based on poor melting curves above this C_t value. Samples with poor expression (1 of 62 cells expressing fewer than 10 of the 42 genes) were eliminated. Expression of housekeeping genes $Actb$ and $Gapdh$ was used to control that RNA had been amplified from the cells, rather than for normalization, in line with recommendations from several studies (e.g. Sindelka et al., 2006). Samples that failed to express both genes were eliminated (4 of 62 cells).

The Mann-Whitney non-parametric U-test was used as a first-line test for comparing E10.5 pancreas and organoids. Statistical distributions after the exclusion of non-expressing cells were tested for normality by the Kolmogorov-Smirnov, Shapiro-Wilk and D'Agostino and Pearson omnibus normality test. Data that were considered to be normally distributed were further tested for statistical differences ($P<0.05$) using an unpaired t-test with Welch's correction, comparing the Ct values of expressing cells.

RESULTS

In vitro pancreas organoid expansion from dispersed progenitors

To identify culture conditions that allow the maintenance and growth of embryonic pancreatic progenitors *in vitro*, we seeded dispersed epithelial cells from E10.5 mouse pancreata at low density in Matrigel (Fig. 1A). In the organogenesis medium that we developed, pancreatic organoids formed with a frequency of 47 organoids/pancreas (i.e. 4-9% efficiency when normalized to E10.5 pancreata of 500-1000 cells) from all the mouse lines that we tested. A reporter line that expresses nuclear GFP under the $Pdx1$ promoter ($Pdx1$/nGFP) (Johansson et al., 2007) provides a convenient way of live-monitoring pancreatic progenitor maintenance with single-cell resolution. The organoids expanded to 3000-40,000 cells in 7 days and the largest 20% (8.4/pancreas) underwent a spectacular morphogenesis reminiscent of the branched pancreas structure (Fig. 1B).

Live monitoring of seeded cells in the first 63 hours of culture (supplementary material Movies 1, 2) revealed proliferation with a doubling time of ~12 hours. Proliferation was still abundant after 7 days of culture (Fig. 2A; pHH3$^+$ cells among total E-cadherin$^+$ cells, 4±1.2%; n=5; supplementary material Fig. S1D for EdU). Organoids became cystic after 10 days in culture and could be maintained for a maximum of 2 weeks. Passaging after mechanical dissociation to smaller clusters or enzymatic cell dispersion led to heterogeneous maintenance of $Pdx1$/nGFP and the rapid formation of cysts (supplementary material Fig. S2). As reported for intestinal organoids (Sato et al., 2009), the BMP inhibitor noggin, supplied either continuously or transiently after reseeding, slowed down the cystic phenotype onset (supplementary material Fig. S2).
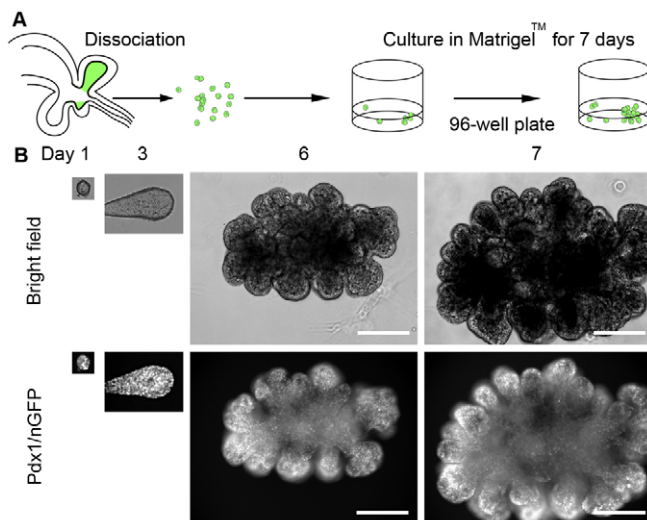
Fig. 1. Expansion and morphogenesis of pancreatic organoids *in vitro*. (**A**) The procedure consists of dispersing progenitors from E10.5 mouse pancreatic buds and seeding them at low density in growth factor-depleted Matrigel. At this stage, the pancreas epithelium is composed of ~90% pancreas progenitors endowed with the ability to differentiate into exocrine and endocrine cells. (**B**) Time-course of the expansion from a small cluster to a branched organoid of ~10,000 cells in organogenesis medium. Phase contrast and fluorescence imaging using nuclear GFP (nGFP) driven by the *Pdx1* promoter marks all pancreatic cells. Scale bars: 200 μm.

Organoids recapitulate pancreas development

Histological characterization of the branched organoids at day 7-10 showed that they were composed of polarized epithelial cells (Fig. 2A,C; supplementary material Fig. S3A) lining a lumen and expressing progenitor markers; whereas most cells expressed both SOX9 and PDX1, HNF1B was restricted to a subset of the cells and was confined to the center of the organoids, similar to what is observed in the pancreas between E14.5 and E18.5 (Fig. 2B,C; representative variation is shown in supplementary material Fig. S1A-C). Based on lineage tracing performed *in vivo*, these *Hnf1b*^{+} cells are expected to be ductal/endocrine progenitors, whereas the peripheral cells expressing *Pdx1* and various levels of *Sox9* are expected to be on their way to exocrine differentiation (Solar et al., 2009; Furuyama et al., 2011; Kopp et al., 2011). Quantitative PCR revealed that organoids at day 7 expressed similar levels of *Pdx1* and *Sox9* to the E10.5 pancreas epithelium (Fig. 3J,M). Single-cell transcript quantification comparing E10.5 pancreatic epithelial cells with those cultured for 7 days confirmed that all cells remained epithelial, expressing increased levels of E-cadherin. Organoid epithelial cells were found to have similar average levels of the pancreas progenitor markers *Pdx1*, *Sox9*, *Hnf1b* and *Nkx2.2* to epithelial cells isolated directly from E10.5 pancreas buds. However, after culture their distribution was broader (including non-expressing cells), most likely revealing the initiation of differentiation (supplementary material Figs S4, S5). By contrast, the increased proportion of mucin 1-expressing cells and their higher average expression confirms the emergence of polarized ducts in culture. We also observed increased levels of *Nkx6.1* and decreased *Mnx1* levels after culture (supplementary material Figs S4, S5). *Mnx1* is expected to decrease after E10.5 *in vivo* (Li et al., 1999) and this event is thus recapitulated *in vitro*.

The exocrine markers PTF1A and amylase were detected in 15-20% of cells located at the periphery of the organoids, forming a partial or continuous crown (Fig. 2D; supplementary material Fig. S1A-C, Fig. S3B). Endocrine cells were observed in a central location, but were rare (0.17±0.09% glucagon + insulin) (Fig. 2E; supplementary material Fig. S6F, Fig. S7F). To test if the progenitors retained a potential to differentiate into endocrine cells after *in vitro* culture, we conducted a functional test in which progenitors grown *in vitro* for 6 days were transplanted into fresh E13.5 pancreatic explants and thus exposed to their native microenvironment (Fig. 2F). Progenitors from *Sox2-Cre; R26R-YFP* mice were used to permanently track the fate of grafted cells. Histological analysis of the explants after 4 and 6 days of culture revealed that the grafted cells had incorporated into the host epithelium either individually or as a group amongst similar host cells (Fig. 2G-J). Grafted progenitors were maintained (Fig. 2G) and proliferated (Fig. 2I), giving rise to both acinar (Fig. 2H) and ductal [positively stained for *Dolichos biflorus* agglutinin (DBA)] (Fig. 2I) cells. In addition, they differentiated into endocrine cells (4.18±5.51%; *n*=8; Fig. 2J). The endocrine cells constitute 2-5% of the total PDX1^{+} population at E14.5 (*n*=3). Taken together, these data demonstrate the efficiency of the organoid culture system for recapitulating normal development in the absence of mesenchyme. This includes pancreatic progenitor maintenance, expansion and differentiation into exocrine lineages. Their endocrine differentiation potential is partially achieved *in vitro* and is fully enabled in the correct niche.

Organoid culture mimics the molecular hallmarks of *in vivo* pancreas development

We investigated whether our culture system mimics *in vivo* development and assessed its utility for the screening of small molecules that might affect pancreas progenitor expansion, differentiation and morphogenesis. Removal of R-spondin 1 (supplementary material Fig. S6), EGF (supplementary material Fig. S7) or phorbol myristate acetate (not shown) did not modify the frequency of cluster formation, cluster size, branching or differentiation but their combined removal reduced the overall culture system efficiency. In the pancreas, fibroblast growth factor 10 (FGF10) from the mesenchyme promotes progenitor maintenance and expansion (Bhushan et al., 2001; Hart et al., 2003; Norgaard et al., 2003). FGF10 in the culture medium reduces acinar differentiation (supplementary material Fig. S8E), whereas FGF1 limits endocrine differentiation (Fig. 6; see below). When neither of the two FGFs was provided, small clusters formed, but they exhibited low/no *Pdx1* expression based on *Pdx1*/nGFP fluorescence (Fig. 3A-D). When endogenous signaling was additionally blocked using the FGF pathway inhibitor SU5402, organoid formation was completely prevented (data not shown). By contrast, blockage after 4 days of culture did not affect organoid expansion or *Pdx1* maintenance (supplementary material Fig. S8A-C), suggesting that organoids become less dependent on FGF signaling after a few days. *In vivo*, *Fgf10* expression decreases after E11.5 (Bhushan et al., 2001) and FGF signaling may be prevalent in early organ expansion.

The Notch signaling pathway promotes the maintenance of pancreatic progenitor properties and their expansion (Apelqvist et al., 1999; Hald et al., 2003; Murtaugh et al., 2003; Magenheim et al., 2011). When Notch signaling was inhibited from the onset of culture with 10 μM DAPT, the Notch signaling target *Hes1* decreased by 60% and organoids were 60% smaller (Fig. 3G-I). DAPT reduced *Pdx1* levels by 25% and halved *Sox9* transcription levels (Fig. 3J,M). Gain-of-function of Notch signaling using the *Gt(ROSA)26Sor*[tm1(Notch1)Dam]*; Pdx1-Cre* mouse line (Murtaugh et al., 2003) did not increase the efficiency of organoid formation in the
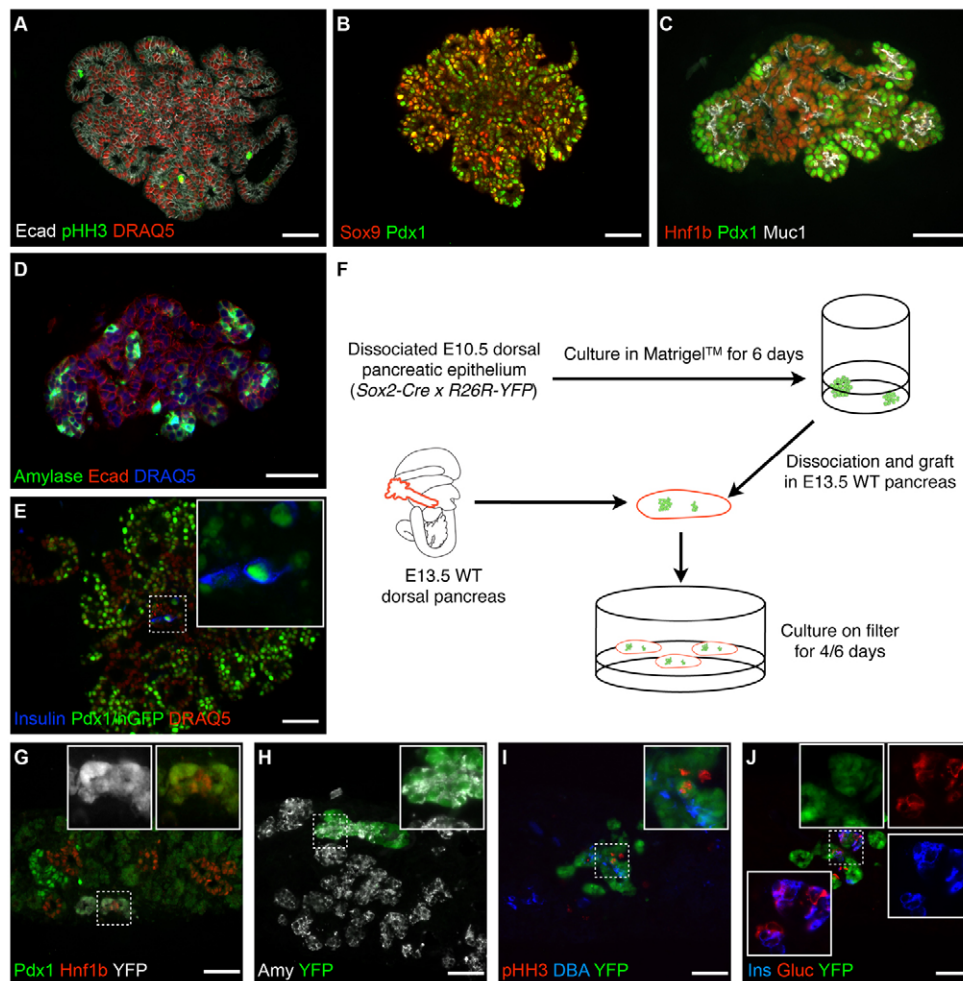
Fig. 2. Organoids recapitulate progenitor expansion and organized differentiation. (**A-E**) Immunohistochemistry on sections of 7-day organoids showing that (A) all cells (DRAQ5, red nuclei) are epithelial (E-cadherin) and many proliferate [phospho-histone H3 (pHH3)] and (B,C) retain pancreatic markers PDX1, SOX9 and HNF1B. (C) Cells polarize and form tubes lined by mucin 1. (D) Exocrine differentiation (amylase) is seen at the periphery. (E) Endocrine differentiation (insulin) is detected in the center. The section in B is close to that in A, and the section in D is close to that in C. (**F**) Experimental scheme to test endocrine differentiation after back-transplantation of cells grown in organoid in a pancreatic niche. WT, wild type. (**G-J**) The cells that were first grown *in vitro* integrate into the host epithelium (white in G and green in H-J). Some remain progenitors (G; HNF1B), some become acinar (H; amylase) or ductal (I; DBA) and others become endocrine (glucagon or insulin). Insets are magnifications of the dashed boxes. Scale bars: 50 μm.

cells that had activated the locus, as monitored by GFP expression (data not shown). Although Notch activity enhances progenitor maintenance and expansion *in vitro* as it does *in vivo*, our experiments reveal that it is not sufficient to elicit clonal organoid formation.

## Pancreas progenitor expansion is dependent on a community

To investigate more precisely the expansion kinetics of dispersed pancreatic progenitors we performed time-lapse analysis on single cells and small clusters of defined cell number (Fig. 4). To be able to form, *Pdx1*/nGFP⁺ organoids required a minimum of four pancreatic progenitor cells in one cluster (Fig. 4A). The frequency of organoid formation and *Pdx1* maintenance rose for clusters of increasing size to reach 100% above 12 cells (Fig. 4A). Among the 158 single cells analyzed, 30% died and 50% lost the progenitor marker *Pdx1*. Of the single cells that retained *Pdx1*, half divided only once (supplementary material Movies 1, 2). This system thus reveals a cooperative effect of cells that is required for the maintenance of their progenitor character and their expansion *in vitro*.

Although the E10.5 pancreas is mainly composed of progenitors, there are already a few NEUROG3⁺ endocrine progenitors and glucagon-expressing endocrine cells at this stage. We investigated whether the apparent cooperativity would reflect the need for an endocrine progenitor in the cluster to trigger proliferation. Indeed, LGR5⁺ gut stem cells form intestinal organoids more efficiently in the presence of cells of the secretory lineage (Sato et al., 2011). Cells from *Neurog3⁻ᐟ⁻* animals formed organoids and proliferated as

efficiently as wild type (Fig. 4C,D). Yet, live imaging revealed that clusters that turned on *Neurog3*/YFP (27% of clusters, *n*=31) during the first 63 hours of culture had a greater chance of forming organoids (43% versus 20%; supplementary material Movie 3). These data argue that endocrine cells are not an essential source of expansion signals but that they promote the process.

## 3D architecture and repression of actin dynamics promote pancreas progenitor maintenance

In our attempts to remove culture components one by one, we observed that the Rho-associated protein kinase (ROCK) inhibitor Y-27632 was an essential component of the culture medium. In the absence of Y-27632, few organoids were formed and none expressed *Pdx1*. Our observations at the early stages of culture revealed that the ROCK inhibitor not only prevents cell death, as previously reported (Watanabe et al., 2007; Harb et al., 2008; Chen et al., 2010), but also maintains *Pdx1*/nGFP expression in the numerous cells that survived (Fig. 4E). ROCK is a kinase that has multiple targets, but its major function is to regulate actomyosin contraction via myosin phosphatase target subunit 1 (MYPT1; PPP1R12A – Mouse Genome Informatics) and myosin regulatory light chain (MRLC) (Amano et al., 2010). Blebbistatin, an inhibitor of myosin II, promotes organoid formation almost as efficiently as Y-27632 (Fig. 4F). This argues that cytoskeletal dynamics, possibly via an impact on cell polarity or through general tensional pre-stress, might control progenitor maintenance. In support of a role of polarity, we observed that the seeded cells, which are not yet polarized in the E10.5 pancreas
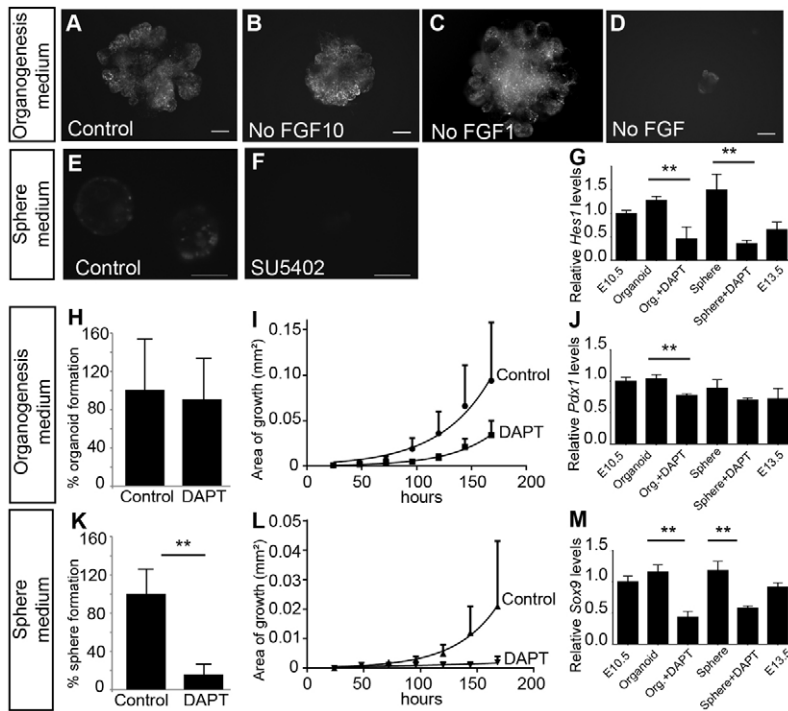
Fig. 3. 3D *in vitro* pancreas organogenesis relies on FGF and Notch signaling, similar to the embryonic pancreas. (**A-F**) Fluorescence imaging of nGFP driven by the *Pdx1* promoter in the presence of FGFs (A-E), after removal of FGF10 (B), FGF1 (C), all exogenous FGF sources (D,F) or inhibition of all FGFs by 10 μm SU5402 (F). (**H**,**K**) Quantification of organoid/sphere formation efficiency in the presence or absence of 10 μm DAPT in organogenesis medium (H) and in sphere medium (K). **P<0.0001, *t*-test, *n*=3. (**I**,**L**) Quantification of the reduced expansion with or without 10 μm DAPT in organogenesis (I) or sphere (L) medium as compared with the condition without DAPT. *P*<0.01, ANOVA, *n*=7 organoids/12 spheres each in two independent experiments. This quantification is estimated by 2D measurements using ImageJ. (**G**,**J**,**M**) Quantification of *Hes1* (G), *Pdx1* (J) and *Sox9* (M) expression levels in the two media with or without 10 μm DAPT. Expression of both *Pdx1* and *Sox9* was reduced significantly in the presence of DAPT. **P<0.05, *t*-test, *n*=3. Error bars represent s.d. Scale bars: 100 μm.

(Kesavan et al., 2009), became polarized during organoid culture. The apical components aPKC and mucin 1 became distributed opposite to the extracellular matrix contained in the Matrigel (Yu et al., 2008) (Fig. 2C; supplementary material Fig. S3A). By 20 days of culture all organoids became cystic and they locally lost mucin 1 and aPKC apical expression, which correlated with a loss of PDX1 and SOX9 expression in these areas (supplementary material Fig. S3C,D).

We also attempted to grow pancreas progenitors on a range of 2D substrates in media that enabled organoid formation in three dimensions. On laminin 1 and gelatin feeder layers, E10.5 pancreatic progenitors could attach and spread but, although they survived and proliferated, they lost *Pdx1* expression within a few days (mostly on day 1) after seeding (supplementary material Fig. S9A). On Matrigel, most cells spread on the matrix but lost *Pdx1* expression, while a
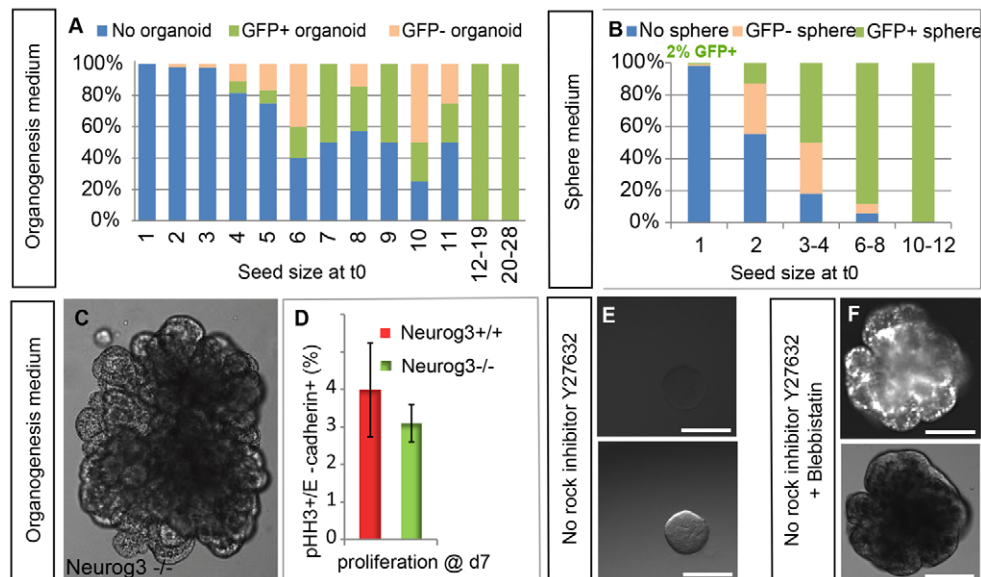
Fig. 4. Community effect and actin dynamics promote 3D *in vitro* pancreas organogenesis. (**A**) Multiplexed imaging for phase contrast and *Pdx1*/nGFP fluorescence from 281 seeds shows the frequency of organoid formation and *Pdx1* maintenance when starting from single cells or clusters of 2 to 28 cells grown in organogenesis medium. (**B**) The same experiment as in A but starting from 223 seeds grown in sphere medium. (**C**) Phase contrast image of a representative organoid from a *Neurog3^{−/−}* mouse. (**D**) Quantification of proliferating epithelial cells in wild-type and *Neurog3^{−/−}* backgrounds. No statistical difference was detected by *t*-test or non-parametric Mann-Whitney U-test, *n*=5 for each. (**E**) Absence of *Pdx1*+ organoid formation in organoid medium depleted of the ROCK inhibitor Y-27632. (**F**) Organoid formation is rescued if 10 μM Blebbistatin is provided in the absence of Y-27632. Scale bars: 100 μm.
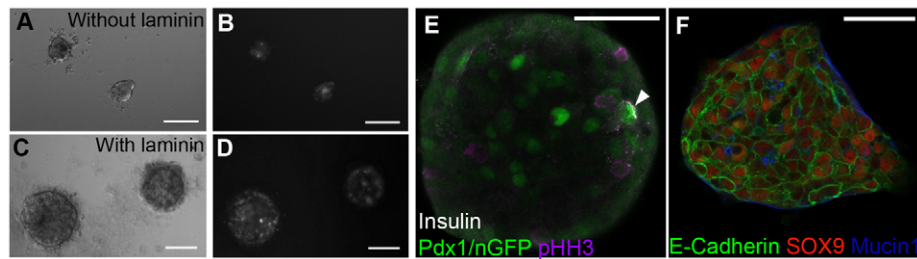
Fig. 5. 3D expansion of pancreas progenitors in defined hydrogels. (**A-D**) Organoids devoid of branches observed by phase contrast (A,C) and fluorescent *Pdx1*/nGFP (B,D) after 7 days of culture in soft PEG with (C,D) or without (A,B) laminin functionalization. (**E,F**) Immunohistochemistry reveals the expression of pancreas progenitor markers PDX1 (E) and SOX9 (F), maintenance of the epithelial marker E-cadherin (F) and internal polarization (mucin 1; F), as well as proliferation (pHH3; E) and occasional endocrine differentiation (insulin; E). Scale bars: 100 μm in A-D; 50 μm in E,F.

fraction invaded the Matrigel and formed organoids (supplementary material Fig. S9B). Taken together, these data suggest that a 3D architecture is needed for optimal progenitor maintenance.

Chemically defined matrices as alternative substrates in drug screening and cell therapy

The *in vitro* culture system that we developed recapitulates the *in vivo* development of the pancreas and can be used to discover regulatory mechanisms that are difficult to uncover *in vivo*. However, the mouse tumor basement membrane-derived matrix might not be ideal for cell therapy and drug screening applications because of regulatory issues (animal product), complexity and batch-to-batch variability. With a view to replacing Matrigel with a fully chemically defined 3D scaffold, several synthetic hydrogel matrices were tested. Although we could not yet identify a synthetic 3D matrix that was as potent as Matrigel at inducing organoid morphogenesis, we observed that pancreas progenitors could be maintained (Fig. 5C-F) and expanded (Fig. 5E) in PEG-based hydrogels (Ehrbar et al., 2007) that were covalently functionalized with laminin 1 (Fig. 5C,D) (0.7-4.1 PDX1$^+$ organoids per 1000 cells seeded, *n*=5 experiments). In non-functionalized hydrogels small

clusters progressively lost their pancreatic and epithelial characters and did not expand (Fig. 5A,B), indicating a necessity for integrin engagement in this process. Moreover, only very soft hydrogels with a shear modulus (G′) of ~250 Pa were able to sustain cluster formation and progenitor maintenance (Fig. 5), unlike stiffer hydrogels (G′>1 kPa, data not shown).
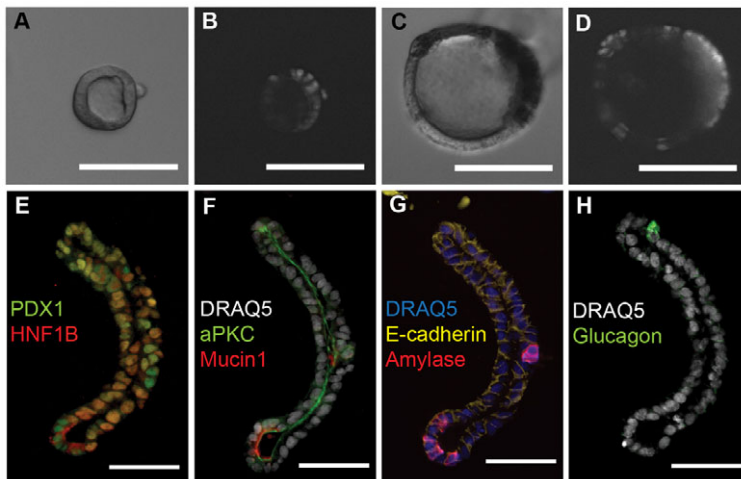
Differentiation of pancreas progenitors into endocrine cells *in vitro*

In the culture conditions described above, endocrine cells differentiate less efficiently than *in vivo*. However, in the absence of FGF1 we observed increased endocrine differentiation, without notable effects on expansion, branching or exocrine differentiation (Fig. 6A-C). Differentiated cells expressed insulin or glucagon but rarely the two together (Fig. 6A,C), and efficient insulin processing was attested by insulin$^+$ cells expressing high levels of PDX1 and prohormone convertase 1/3 (PC1/3; PCSK1 – Mouse Genome Informatics) and exhibiting high levels of C-Peptide1/2 (Fig. 6D-E″). NEUROG3 protein was also detected (Fig. 6F). Inhibition of FGF signaling by SU5402 after 4 days also triggered differentiation (supplementary material Fig. S8D).
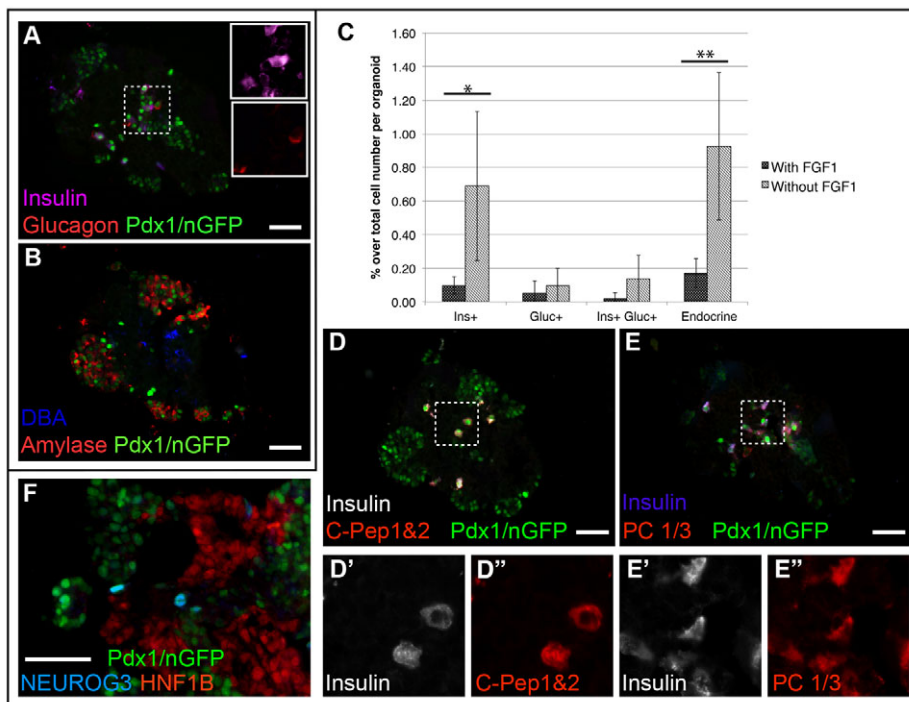
Fig. 6. Increased endocrine differentiation in organoids upon FGF1 depletion. (**A**) Immunohistochemistry of organoids grown in the absence of FGF1. Increased endocrine differentiation (insulin, glucagon) is seen after 7 days of culture. In all panels, the cells with high levels of *Pdx1*/nGFP show the exocrine crown that surrounds organoids. (**B**) Normal exocrine differentiation (amylase, DBA). (**C**) Quantification of insulin$^+$, glucagon$^+$ and double-positive cells in the presence or absence of FGF1. Error bars indicate s.d.; *P*=0.003, **P*=0.0008, Mann-Whitney non-parametric U-test, *n*>4. (**D-E″**) Immunohistochemistry shows that the insulin$^+$ cells co-express high levels of *Pdx1* (D) and prohormone convertase (E-E″) and exhibit high levels of C-peptide (D-D″). (**F**) Endocrine progenitors (NEUROG3) are detected close to central progenitors expressing HNF1B. Scale bars: 50 μm.

Fig. 7. The maintenance of pancreas progenitors is more homogenous in spheres. (**A-D**) Culture in sphere medium promotes the formation of hollow spheres as observed by phase contrast or *Pdx1*/nGFP, in this case after 4 (A,B) and 7 (C,D) days of culture. (**E-H**) After treatment for immunohistochemistry, the monolayered spheres collapse. Immunohistochemistry reveals the maintenance of progenitor markers PDX1 and HNF1B (E). Cells remain epithelial (G) (E-cadherin) and acquire polarity, with their apical side facing the lumen (mucin 1 and aPKC; F). A small subset of cells differentiates into endocrine (glucagon; H) and exocrine (amylase; G) cells. DRAQ5 marks all nuclei. Scale bars: 100 µm in A-D; 50 µm in E-H.

Culture conditions to promote efficient and homogeneous progenitor expansion

The organogenesis system presented above recapitulates the developmental balance between expanding progenitors and differentiating cells but is not ideal for homogenous progenitor expansion. By contrast, dispersed E10.5 pancreas cells grown in Matrigel in a medium that was previously shown to promote the maintenance of human embryonic stem cell (hESC)-derived $PDX1^+$ cells (Ameri et al., 2010) formed hollow spheres with a frequency of 60 spheres/pancreas (i.e. ~6-12% efficiency when normalized to E10.5 pancreata of 500-1000 cells) (Fig. 7A,B). The spheres, which were 100-800 µm in diameter (Fig. 3L, Fig. 7C,D), were outlined by a monolayer of $PDX1^+$ (96% of 834 cells), $SOX9^+$ (all) and $HNF1B^+$ (72% of 834 cells, 68% co-expressing PDX1 and HNF1B) cells interspersed with rare endocrine (1.56%) and exocrine (5.73%) cells after 7 days in culture (Fig. 7E-H). *Sox9* and *Pdx1* expression levels were similar to those of E10.5 pancreata (Fig. 3J,M). These spheres expanded (Fig. 3L; supplementary material Movie 4) and could be passaged at least twice. As in the organogenesis conditions, sphere formation, progenitor maintenance and expansion relied on FGF and Notch signaling (Fig. 3E-M).

Time-lapse imaging revealed that, as in the organogenesis conditions, there was a cooperative effect by which groups of increasing numbers of cells formed expanding spheres more efficiently (Fig. 4B). However, in contrast to the organoid culture system, spheres were able to form from single cells with an occurrence of 2%. Groups of two and four cells had a 50% and 90% chance of forming a sphere, respectively (Fig. 4B). The sphere medium is thus well suited to maintain a more homogeneous population of progenitors and minimize their differentiation.

DISCUSSION

Our experiments have established 3D culture protocols that are valuable to model pancreas development *in vitro* and to test parameters that might be difficult to manipulate *in vivo*. We have used these culture systems to unravel novel aspects of pancreas development, such as the necessity of a 3D environment for progenitor expansion as well as a 'community effect'. The recapitulation of pancreatic morphogenesis *in vitro* will be useful to systematically address a wealth of questions concerning the regulation of morphogenetic processes but also differentiation in a physiologically relevant 3D context (Kesavan et al., 2009). Importantly, our work paves the way for preclinical and clinical applications of cells generated *in vitro*, such as the expansion of

pancreas progenitors from stem cells for diabetes therapy and drug screening.

Recently, the formation of intestinal organoids, optic cups and cerebral organoids from ESCs has been achieved (Eiraku et al., 2011; Spence et al., 2011; Lancaster et al., 2013). Our 3D culture conditions could potentially optimize the expansion of pancreatic progenitors produced from hESCs (Ameri et al., 2010; Cheng et al., 2012). This will be an essential step toward developing effective clinical applications for *in vitro* generated beta cells. Progenitor expansion in the sphere medium maintained the greatest homogeneity and exhibited the longest expansion time (at least 3 weeks). However, the culture duration will need to be improved for production purposes. In sphere and organogenesis media we have measured a 50-fold expansion of the original population in 7 days, with smaller but more numerous formations in sphere conditions. If the seeding of those cell clusters with an initial population that provides the highest expansion probability could be achieved reliably, then, in principle, progenitor expansion efficiency should improve. For production purposes, the heterogeneity of the organogenesis conditions is not optimal due to the differentiation of many exocrine cells. More generally, the diversity created is likely to lead to complex endogenous signaling between cells, limiting control over the production process. Nevertheless, we demonstrate that progenitors (1) retain their properties after culture, (2) are able to contribute to ducts and (3) can differentiate when reseeded in pancreata.

As an *in vitro* model of development, we show that the organogenesis system strikingly recapitulates many aspects of pancreas development *in vivo*, including morphogenesis and differentiation. The cells differentiate at the proper location in the organoid akin to those in the pancreas, with acini at the periphery and ducts and endocrine cells in the center. Although acini form, the degree of maturity of the exocrine cells, which normally express increasing levels of exocrine enzymes, has not been determined. However, the endocrine differentiation efficiency remains lower than *in vivo* and even under optimized conditions only 1% endocrine differentiation is achieved. Although glucagon- and insulin-producing cells with several functional characteristics are formed, the presence of other endocrine cell types remains to be investigated. The system also recapitulates the process of duct formation. In a population of initially non-polarized cells, mucin 1 expression is turned on and polarity proteins are targeted in a directional manner to form ducts. Thus, the system might provide an extremely valuable way to further study the acquisition of

polarity in a simplified setting. In this respect, we show that the inhibition of ROCK or its downstream target myosin II is needed to promote dissociated cell survival (Watanabe et al., 2007) and to maintain progenitor properties. The ROCK pathway controls microfilament dynamics and our experiments show that too much tensile stress is detrimental to *Pdx1* maintenance and to progenitor expansion. Changes to microfilament dynamics may result in cell polarization defects or may affect the formation of junctions, as has been reported in other systems (Itoh et al., 2012; Rodríguez-Fraticelli et al., 2012). It is possible that *Pdx1* transcription might in turn rely on polarity and cell-cell interactions. We have observed that *Pdx1* is better maintained when cells are clustered and tends to be lost in areas lacking polarity. How two different culture medium compositions promote different structural outcomes and the causal links between structure and differentiation remain to be addressed.

In the embryonic pancreas after a phase of expansion, a reduction of FGF activity is required to increase endocrine cell differentiation (Kobberup et al., 2010). We show that pancreatic organogenesis *in vitro* relies on both FGF and Notch pathway activity, as it does *in vivo* (Apelqvist et al., 1999; Jensen et al., 2000; Bhushan et al., 2001; Hart et al., 2003; Norgaard et al., 2003). However, although activation of Notch promotes pancreas progenitor maintenance (Hald et al., 2003; Murtaugh et al., 2003), it is not sufficient to promote the formation of organoids from single cells. The ability to form organoids is unlikely to depend solely on a requirement for endocrine or NEUROG3^{+} cells to express Notch ligands (Sato et al., 2011). Indeed, *Neurog3* inactivation did not alter organoid formation in the pancreas. In line with this, it was recently shown that *Dll1* is expressed in all multipotent pancreatic progenitors at E10.5 (Ahnfelt-Rønne et al., 2012). Thus, potentially any of the epithelial cells in the early pancreas could sustain Notch activity and in turn promote pancreas progenitor maintenance in neighboring cells. This hypothesis is, however, in contrast to the Notch-dependent interaction observed specifically between Paneth cells and intestinal stem cells (Sato et al., 2011). Moreover, synergistic Notch activity might underlie the community effect.

Interestingly, organogenesis in the conditions that we have defined occurs in the absence of mesenchyme and of endothelia that normally sustain pancreas development (Lammert et al., 2001; Landsman et al., 2011). However, at least two mesenchymal factors are provided: FGFs and laminin. The latter is the most abundant component in Matrigel and might initiate polarity acquisition. The importance of laminin is confirmed in the more defined conditions of the synthetic hydrogels. However, we cannot discount the fact that other mesenchymal niche factors potentiate endocrine cell differentiation.

In contrast to the organoid medium, which provides a system that best mimics pancreas development *in situ*, the sphere medium has the advantage of clonal growth. In future experiments we hope to combine the clonal culture of pancreas progenitors with cell sorting to analyze defined progenitor cell populations at different stages of development. They share similarities with the spheres recently published by Sugiyama et al. (Sugiyama et al., 2013). By using methods such as multiplexed single-cell gene expression analysis one should be able to provide a paradigm to test the response of single cells to the perturbation of a specific signaling pathway or to changes in extracellular components. The complementarity of the organoid and sphere systems that we have developed, together with adaptations to human pluripotent stem cells and developments in gene interference, will open new avenues in diabetes research and therapy.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.096628/-/DC1

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