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 requires 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 the 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+ CD49fhigh 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 4×106) 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 miniorgs 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-L-Ngn3-ER_TM-ires-nGFP (Johansson et al., 2007); Sox2-Cre (Hayashi and McMahon, 2002); R26R-YFP (Srinivas et al., 2001); R26R-IacZ (Komada and Soriano, 1999); Ngn3-EGFP (Mellitzer et al., 2004); Gt(ROSA)26Sor1m1(Nos1)Rtm1(Murtagh 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 Minitray (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-Ménéur 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 M 2-mercaptoethanol; 16 mM phosphor 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 C1 strain and from the Ngn3/EGFP/+; Sox2-Cre x R26R-IacZ; Sox2-Cre x R26R-YFP and Pdx1-L-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 lab), 10 µM SU5402 (Calbiochem) stocks at 10 mM in DMSO were diluted to 10-40 µM. A 1:250 dilution of DMSO was added to all final medium compositions.

In vitro generated minipancreas

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.

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 (NQEQLSPL-ERG-CNH2) and lysine-containing peptides with MMP-sensitive sequences: AcFKGG-GPGIWGQ-ERG-CNH2. 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 XIIa 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 (F 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.

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; Biotium) 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-ERTM-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, CO2-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 and dispersed as described above. Individual cells were handpicked by 95°C for 2 minutes, then underwent 50 cycles of PCR amplification (Applied Biosystems). Samples were heated to 50°C for 2 minutes followed by inactivation at 95°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 expression, based on poor melting curves above this Ct 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 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. Passingage 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 seeding, slowed down the cystic phenotype onset (supplementary material Fig. S2).
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 day 7 expressed similar levels of Pdx1 promoter marks all pancreatic cells. Scale bars: 200 μm.

Organoids 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 Git(Rosa)26Sox^{tm1(Notch1)Dam}; Pdx1-Cre mouse line (Murtaugh et al., 2003) did not increase the efficiency of organoid formation in the partial or continuous crown (Fig. 2G; 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 among 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.

**Organoids culture mimics the molecular hallmarks of in vivo pancreas development**
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−/− 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; PPPIR12A – 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...
(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...
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).
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. 3L,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 pancreas.

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; Rodriguez-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 NEUROG transgenic organoids (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-Ronne 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 extracellular component. 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.

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Author contributions
C.G., F.D.F., M.F.-L., S.G., A.R., H.S., M.L. and A.G.-B. designed experiments and wrote the manuscript. C.G. and F.D.F. performed most experiments. M.F.-L. performed experiments leading to Figs 3G-M and Figs S4 and S5. S.G., A.R. and M.L. provided expertise in bioengineering leading to Fig. 5.

Supplementary material
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References


In vitro generated minipancreas


