Ex Vivo Expansion and Differentiation of Human and Mouse Fetal Pancreatic Progenitors Are Modulated by Epidermal Growth Factor

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A comparative analysis of mouse and human pancreatic development may reveal common mechanisms that control key steps as organ morphogenesis and cell proliferation and differentiation. More specifically, understanding beta cell development remains an issue, despite recent progress related to their generation from human embryonic and induced pluripotent stem cells. In this study, we use an integrated approach, including prospective isolation, organ culture, and characterization of intermediate stages, and report that cells from human and mouse fetal pancreas can be expanded in the long term and give rise to hollow duct-like structures in 3D cultures. The expanded cells express a combination of markers (E-cadherin, PDX1, NKX6-1, SOX9, and HNF1β) that reveals pancreatic progenitor identity. Proliferation of embryonic progenitors was stimulated by the Wnt agonist R-spondin1 (RSPO1), FGF10, and EGF. This combination of growth factors allowed maintaining human fetal pancreatic progenitors in culture for many passages, a finding not reported previously. Importantly, in the absence of EGF, proliferation was reduced, while endocrine differentiation was significantly enhanced. We conclude that modulation of EGF signaling affects in vitro expansion and differentiation of progenitors from embryonic pancreas of both mice and man.

Introduction

Pancreatic development has been extensively studied in rodents and lower vertebrates, and a systematic analysis of human pancreatic development, including early bud formation, has been recently reported [1]. Direct comparison of the response of human and rodent pancreatic progenitors to signaling molecules under defined conditions would be the next step of progress. The characterization of human pancreatic progenitors in vivo and the definition of their identity in vitro would represent important tools to better understand and model ex vivo organ development, offering a new tool to dissect the molecular control of cell fate choice in humans and corroborate data obtained in rodent model systems.

Recently, both embryonic and adult murine pancreatic progenitors have been expanded in 3D culture [2–5]. Following in vitro expansion, pancreatic progenitors from adult mice were transplanted under the kidney capsule and expressed beta cell differentiation markers [2]. Moreover, mouse embryonic progenitors from the very early embryonic stage (E10.5) recapitulated organ development in vitro, including branching and acinar/islet morphogenesis [3].

In addition, progenitors from E11.5 mouse embryos could be sorted as single cells and recapitulated endocrine differentiation in vitro [5]. However, developing model systems for screening the effect of signaling factors on expandable pancreatic progenitors to direct endocrine cell differentiation that could be translated to humans remains a major objective, also in relation to recently developed embryonic stem (ES) and induced pluripotent stem-derived beta cells [6,7]. Multipotent progenitors isolated from developing human and mouse pancreases and characterized by the expression of specific transcription factors, such as PDX1, NKX6-1, SOX9, HNF1β, and PTF1A [8–11], would represent a good cell source for optimizing conditions to achieve this goal. In human pancreas, PDX1 and SOX9 are extensively expressed by early progenitors at the stage of ventral and dorsal pancreatic bud formation [30–33 days postconception (dpc)], while NKX6-1 is detected in the dorsal bud and NKX2-2 still is absent. From 47 dpc onward, PDX1, SOX9,
and NKX6.1 are expressed in tip and trunk progenitors, while NKX2.2 and insulin appear only in fetal beta cells starting at 8 weeks postconception (wpc) following transient expression of NGN3 [1].

We aimed to assess in vitro expansion and endocrine differentiation potential of human and mouse progenitors, isolated at 8–11 wpc and E12–E13, respectively. We provide evidence that efficient amplification of human and mouse fetal pancreatic progenitors can be obtained under 3D culture conditions initially developed for expansion of adult mouse pancreas [2] and show initiation of endocrine differentiation by these progenitors following manipulation of the expansion medium. Importantly, EGF promotes cell proliferation at the expense of beta cell differentiation under these experimental conditions, thus offering a tool to control the extent of proliferation and differentiation.

Materials and Methods

Isolation and dissociation of human fetal pancreas

Pancreases were dissected from human fetal tissue fragments obtained immediately after abortion between 8 and 11 wpc, in compliance with the bioethics legislation in France. Tissue collection and experiments were conducted with the approval of the Agence de Biomedecine (Approval number, PFS08-011). Gestational age was determined by morphological criteria as previously described [12]. Mesenchyme surrounding the pancreas was removed and epithelial tissue was then digested by collagenase IV (250 U/mL; Worthington) in Hank’s balanced salt solution (HBSS; Invitrogen) at 37°C for 20 min. Further dissociation was done by gentle pipetting. Cell clusters were rinsed once with 4 mL of advanced Dulbecco’s modified Eagle's medium/nutrient mixture F12 (AdDMEM/F12; Invitrogen) containing 10% fetal calf serum (FCS) and several times with Matrigel (diluted 1:10 in AdDMEM/F12). After 24 h, cells had attached to the Matrigel and formed colonies that grew as 3D structures.

Mouse strains

All mouse experiments were conducted according to the Ethics Committee for Animal Experiments at the VUB. The double transgenic mouse strain, Pdx1eGFPIns1mRFP, was generated by crossing Pdx1eGFP and Ins1mRFP mice. Pdx1eGFP mice were obtained from Doug Melton (Harvard University, USA) [13]; Ins1mRFP mice were provided by Gerard Gradwohl (IGBMC, Strasbourg, France) and were previously described [2, 14, 15]. OF1 mice for wild-type (WT) explant isolation were purchased from Janvier Labs. All animals were maintained in a 12-h light cycle and provided food and water ad libitum.

Isolation and dissociation of mouse fetal pancreas

Embryonic pancreases were dissected under sterile conditions from Pdx1eGFPIns1mRFP or WT pregnant females at E12 or E13 of gestation. Fetal pancreases were digested by collagenase XI (1.4 mg/mL; Sigma) in isolation medium (Lonza) at 37°C for 7 min combined with mechanical dissociation by gentle pipetting. Small aggregates of ~3–20 cells were resuspended in AdDMEM/F12. For single-cell dissociation, the aggregates were resuspended in dissociation medium (Lonza) containing trypsin (1 mg/mL; Sigma) and DNase (0.4 mg/mL; Sigma) until single-cell suspension was obtained. Trypsin was inactivated by adding equal volume of HBSS containing 10% FCS.

Human and mouse progenitor bulk cell culture

The culture medium contained AdDMEM/F12 supplemented with B27 (Invitrogen), N2 (Invitrogen), 1.25 mM N-acetylcysteine (Sigma-Aldrich), 10 nM nicotinamide (Sigma-Aldrich), 10 nM gastrin (Sigma-Aldrich), 50 ng/mL noggin (Peprotech), 50 ng/mL FGF10 (Peprotech), 500 ng/mL R-spondin (R&D), 50 ng/mL EGF (Peprotech), and 10 μM Rho-associated protein kinase (ROCK) inhibitor Y (Y-27632; Sigma-Aldrich) [2]. A volume of 500 μL of culture medium was added to the 30 μL Matrigel gel containing the progenitor cells. The medium was changed twice a week. Cell clusters were passaged once a week by resuspension in 10 mL AdDMEM/F12, and then pelleted for subsequent mechanical dissociation. The dissociated cells were pelleted once more and transferred to fresh Matrigel. Dilution at each passage was at a 1:4 to 1:6 ratio for human cells and 1:8 for mouse cells.

To assay for the impact of growth factors on cell proliferation and differentiation, half of the newly dissociated cells from single fetal human pancreas or individual mouse explants from the same litter were cultured in the complete medium described above and the other half in the medium depleted for EGF, FGF10, or R-spondin.

Prospective isolation of mouse pancreatic progenitors

Single-cell suspension from Pdx1eGFPIns1mRFP or wild-type mice at E12 or E13 was resuspended in HBSS (Invitrogen) containing 10% FCS for fluorescence-activated cell sorting (FACS). Cells were incubated with anti-mouse EpCAM/APC antibody (eBiosciences) for 30 min on ice, then resuspended in a solution containing propidium iodide (PI, 1 mg/mL; Sigma) and N-(6-methoxy-8-quinolyl)-p-toluene-sulfonamide (TSQ, 1 mg/mL; Molecular Probes), and sorted on an FACSARia (Becton Dickinson). Pdx1eGFP+EpCAM+ were sorted and purity of the enriched population was determined: no endocrine (TSQ+ or RFP+) cells were detected. Pulse-width gating excluded cell doublets, while dead cells were excluded by gating on the PI-negative cells. Sorted populations were embedded in Matrigel, plated in individual 24 wells at different concentrations (2×102–5×105), and cultured in complete medium. Cells were split once a week by dissociation to single cells and counted; for expansion, cells were plated at a density of 15–20×103 per well.

2D cultures of human pancreatic cells

After 1 month of expansion in Matrigel in 3D, organoids of human fetal pancreatic cells were dissociated with trypsin (Sigma-Aldrich) at 37°C for 5 min. Trypsin was inactivated by the addition of 20% serum-containing medium. Following extensive washing, the cells were seeded onto 24-well plates or 8-well microscope slides (Ibidi; Biovalley) coated with Matrigel (diluted 1:10 in AdDMEM/F12). After 24 h, cells had attached to the Matrigel and formed colonies that grew as monolayers. Five to 7 days later, the cells were fixed for 1 h in 4% paraformaldehyde and used for immunocytochemistry.
Protein analysis

Organoids were fixed in 3.7% formaldehyde before embedding them in paraffin. For immunohistochemistry, sections of 4 µm were prepared and processed, as described previously [16]. The following antibodies were used for immunostaining; mouse anti-insulin (1:1,000; Sigma); guinea pig anti-insulin (1:5,000; Diabetes Research Center—VUB); rat anti-human C-peptide (1:3,000; Beta Cell Biology Consortium); mouse anti-glucagon (1:2,000; Sigma); rabbit anti-somatostatin (1:500; DakoCytomation); mouse anti-chromogranin A (1:50; DakoCytomation); rabbit anti-synaptophysin (1:50; Serotec); rabbit anti-human PDX1 (1:2,000) [17], guinea pig anti-PDX1 (1:1,000; Beta Cell Biology Consortium), mouse anti-PDX1 (1:2,000; Developmental Studies Hybridoma Bank); rabbit anti-NKX6-1 (1:500; Beta Cell Biology Consortium), goat anti-NKX6.1 (1:2,000; R&D); sheep anti-human NGN3 (1:400; R&D Systems); mouse anti-NKX2-2 (1:50; Developmental Studies Hybridoma Bank), mouse anti-E-cadherin (1:100; BD Biosciences), rabbit anti-SOX9 (1:500; Millipore), rabbit anti-sox9 (1:100; Chemicon); Armenian hamster anti-Mucin1 (1:500; Thermo Scientific); mouse anti-human Kif6 (1:50; DakoCytomation), rat anti-Ki67 (1,500; eBio- sciences); rabbit anti-pancystokeratin (1:700; Dako); and rat anti-EpCAM-APC (1:400; eBiosciences).

The secondary antibodies were (human experiments) as follows: Alexa Fluor® (AF) 488 goat anti-rabbit (1:400; Invitrogen); AF 594 goat anti-rabbit (1:400; Jackson Immuno Research), AF 594 goat anti-mouse (1:400; Jackson Immuno Research), and biotinylated rabbit anti-sheep (1:200; Vector Laboratories). For mouse experiments, the following secondary antibodies were used: AF 488, 647, and Cy5® (1:500; Jackson Immuno Research), rabbit anti-rabbit (1:500; Jackson Immuno Research), anti-guinea pig (1:500; Jackson Immuno Research), anti-rat (1:500; Jackson Immuno Research), and anti-Armenian hamster (1:500; Jackson Immuno Research). For NGN3, revelation was performed using the Vectastain ABC kit (Vector).

Image acquisition and analysis

Cultivated human cells were imaged using a Leica DMIL microscope and DFC420C camera, while for mouse cells, a Nikon TE2000 inverted automated fluorescence microscope with motorized table and controlled by the NIS elements AR software was used.

Immunofluorescence images of human cells were acquired using an upright Zeiss Axioplan2 fluorescence microscope with a Hamamatsu C10600 ORKA-R2 camera or a confocal microscope (SP5; Leica). A confocal multiphoton Zeiss LSM710 NLO with the TiSa laser microscope was used to acquire immunofluorescence images of mouse cells. Images were analyzed using Leica LAS AF Lite software or Smartcapture 3 (human cells), while confocal images of mouse cells were processed using Improvision Velocity LE and Zeiss Zen software.

RNA analysis

Total RNA was isolated using the QIAGEN RNeasy Micro Kit (QIAGEN) or TRIzol reagent (Invitrogen).

<table>
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<th>Gene name</th>
<th>Gene symbol</th>
<th>Applied Biosystem reference</th>
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Quantitative polymerase chain reaction was performed with cDNA synthesized on 200ng total RNA using species-specific primers and probes from Applied Biosystems as shown in Table 1 and from Applied Biosystem and Integrated DNA Technologies (IDT) as shown in Table 2. Values are represented as mean±standard deviation (human data) or mean±SEM (mouse data). Statistical analysis was performed using an unpaired Student’s t-test. P<0.05 was considered as statistically significant. Data from at least four independent experiments were used.

Results

In vitro expansion of fetal human pancreatic cells

To amplify fetal human pancreatic progenitors, we applied culture conditions that allowed expansion of progenitor cells from adult mouse pancreas [2]. Pancreases of human fetuses at 8–11 wpc were dissected and partially digested with collagenase. The resulting cell aggregates were then embedded in Matrigel and cultured in medium containing EGF, FGF10, R-spondin, and the ROCK inhibitor, Y-27362 (Fig. 1A). Budding cyst-like organoids appeared within 24 to 48 h and were diluted 4- to 6-fold at weekly passages. The expanding cells formed organoid structures, the majority of which were cystic, while a minority were filled with cells. Organoid morphology remained unchanged during expansion (Fig. 1B) for at least 5 months and freeze/thaw cycles did not affect their growth efficiency (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). Cells from more than 80% independent human fetal pancreases were expanded under these conditions at a 100% success rate, supporting robustness of the protocol.
Gene name | Gene symbol | Applied Biosystem or IDT reference
--- | --- | ---
Chromogranin A | Chga | Mm.PT.56a.41352258 —IDT
Cyclophilin A | CyA | Mm02342429_g1
Glucagon | Gcg | Mm004001712_m1
Insulin I | Ins1 | Mm01950294_s1
Leucine-rich repeat-containing G-protein-coupled receptor 5 | Lgr5 | Mm00438890_m1
Neurogenic differentiation 1 | NeuroD | Mm01280117_m1
Neurexin3 | Ngn3 | Mm00437606_s1
NK2 transcription factor related, locus2 | Nkx2-2 | Mm00839794_m1
NK6 homeobox 1 | Nkx6.1 | Mm.PT.56a.28634562 —IDT
Paired box protein 4 | Pdx4 | Mm01159043_g1
Pancreatic and duodenal homeobox 1 | Pdx1 | Mm00435565_m1
Pancreas-specific transcription factor, 1a | Ptf1a | N018809.1.Ptf1a—IDT
SRY-box9 | Sox9 | Mm00448840_m1
Secreted phosphoprotein 1 | Spp1 | Mm.PT.56a.43709208 —IDT
Somatostatin | Sst | Mm00436671_m1

**Fetal human pancreatic organoids express progenitor cell-specific transcription factors**

After 4 weeks of expansion in 3D culture, the majority of the cells were epithelial, as demonstrated by their expression of E-cadherin (Fig. 1C and Supplementary Fig. S2). They also expressed key pancreatic progenitor markers, such as SOX9, PDX1, and NKK6-1 (Fig. 1C). Half of the cells in fetal pancreatic organoid structures (52.2% ± 6.9%; n = 4 independent cultures) were Kif6+, indicating active proliferation (Fig. 1C and Supplementary Fig. S3). In addition, the cells were organized in duct structures and were polarized as demonstrated by MUC1 expression (Supplementary Fig. S3).

To study the cell organization in culture, the growing organoids in 3D were plated on Matrigel in 2D and fixed after adherence. Immunohistochemistry revealed that epithelial PDX1+ cell clusters were surrounded by vimentin+ cells both in 2D and in 3D cultures (Supplementary Fig. S3). The epithelial cells expressed pancreatic progenitor marker proteins, NKK6-1, PDX1, SOX9, and HNF1β, and did not express NKX2-2 or NGN3 (data not shown). Only very rare cells expressed chromogranin-A and/or glucagon, insulin, or somatostatin (Fig. 1D). Epithelial cells that express pancreatic progenitor markers thus can be expanded from fetal human pancreas, but under the above culture conditions do not differentiate to a significant extent. Importantly, expanded cells maintained a stable phenotype throughout long-term culture and after freeze/thaw cycles (Supplementary Fig. S4).

**EGF modulates proliferation and differentiation of fetal pancreatic progenitors**

When ROCK inhibitor was removed from the culture medium, cell proliferation dramatically decreased. Removal of either FGF10 or R-spondin resulted in slower proliferation, but still allowed expansion for at least 10 passages without any differentiation (data not shown).

The most striking effect was noticed by exclusion of EGF from the culture medium. Without EGF, all of 20 independent pancreases tested still expanded to cystic organoids, but could be diluted only 1:2 at weekly passages that were limited to 10. The resulting organoids were smaller and often filled with cells (Fig. 2A, B). After 28 days under these conditions, the majority of the cells were PDX1+, NKX6-1+, SOX9+, HNF1β+, and E-cadherin+ (Fig. 2B), similar to cells cultured in the presence of EGF. However, at variance with cultures in EGF, NKX2.2+ and NGN3+ cells were present (Fig. 2B). In addition, when cells were plated on Matrigel-coated plates to facilitate analysis, many cells expressed the proendocrine marker, chromogranin-A, and endocrine cell markers, such as insulin, somatostatin, or glucagon (Fig. 2C). The vast majority (about 75%) of insulin+ cells were glucagon+ (Fig. 2C). Insulin+ cells were PDX1+ and NKX6-1+ (Fig. 2C).

To quantitatively evaluate endocrine differentiation, comparative quantitative reverse transcription PCR was performed on RNA isolated from fetal human pancreatic cells expanded for 28 days with or without EGF (Fig. 3). The abundance of transcripts coding for PDX1 and NKX6-1 was similar under both conditions. This was also the case for SPP1, a duct cell marker, and for LGR5 and SOX9 as we previously described in long-term expanding of adult mouse duct progenitors, where LGR5+ single cells sustain the growth of organoids with a duct phenotype [2]. On the other hand, the expression of PTF1A, a pancreatic progenitor and mature acinar cell marker, was 10- to 50-fold higher in cultures performed in the absence than in the presence of EGF. Interestingly, the expression level of the endocrine markers, NGN3, NEUROD, NKX2-2, PAX4, chromogranin-A, insulin, glucagon, and somatostatin, was significantly increased in cells cultured in the absence of EGF.

**Prospectively isolated fetal epithelial cells give rise to endocrine cells in culture**

The above data strongly suggest that human fetal progenitors are facilitated to differentiate toward endocrine fate in the absence of EGF. However, in human cells, we could not strictly monitor the dynamics of the differentiation process due to the lack of promoter-specific expression of reporter proteins. On the contrary, in mouse, it is possible to monitor differentiation through activation of promoter-specific fluorescent proteins thanks to available genetic models. Moreover, a direct study of the fate of prospectively isolated fetal epithelial cells was possible in these mice.

Therefore, we first examined the dynamics of expansion and differentiation of fetal progenitors using cells isolated from E12 and E13 Pdx1+EGFPIns+GFP reporter mice embryos. These reporter mice express eGFP under the control of the Pdx1 promoter in early progenitors and in beta cells and mRFP
FIG. 1. In vitro amplification of human pancreatic progenitors isolated from human fetal pancreas can be grown in vitro. (A) Schematic representation of the experimental procedure: human fetal pancreases (8 to 11 weeks of development) were enzymatically and mechanically dissociated. The resulting aggregates were embedded in Matrigel, seeded, and cultured in culture medium containing R-spondin, FGF10, and EGF (complete medium). (B) Photographs of pancreatic aggregates cultured in Matrigel for different periods. Scale bar: 250 μm. Note that with time in culture, predominantly cystic-like structures not only develop but also some are full organoids. Aggregates were dissociated and passaged weekly and kept in culture for more than 6 months with stable morphology. (C) Cell aggregates at passage 4 (28 days in culture), were fixed, embedded in paraffin, sectioned, and stained with anti-SOX9, anti-E-cadherin, anti-PDX1, anti-Ki67, and anti-NKX6-1 antibodies. Scale bars: 50 μm. (D) Organoids at passage 4 (28 days in culture) were dissociated and seeded in Matrigel-coated plates for culture in 2D. Five to 7 days later, cells were fixed and stained with anti-SOX9, anti-PDX1, anti-E-cadherin, anti-HNF1β, anti-NKX6-1, anti-chromogranin-A antibodies, or a mix of anti-insulin, anti-somatostatin, and anti-glucagon antibodies (anti-endocrine cells). Scale bars: 25 μm.
under the control of the insulin promoter. Small cell aggregates of 3 to 20 cells from collagenase-digested fetal pancreases were embedded in Matrigel and cultured in the complete medium, from which EGF was removed. (A) Both cysts and organoids develop under such conditions and can be passaged. Scale bars: 250 μm. (B) After 4 passages, cell were fixed, embedded in paraffin, sectioned, and stained with anti-PDX1, anti-SOX9, anti-E-cadherin, anti-NKX6-1, anti-NKX2.2, and anti-NGN3 antibodies. Scale bars: 50 μm. (C) After 4 weeks in culture, cell aggregates were dissociated using trypsin and seeded in Matrigel-coated plates for culture in 2D. Five days later, cells were fixed, and stained with anti-SOX9, anti-PDX1, anti-E-cadherin, anti-NKX6-1, anti-chromogranin A (CHGA), anti-insulin, anti-somatostatin (SST), and anti-glucagon (GCG) antibodies. Scale bars: 50 μm.

FIG. 2. Amplification and differentiation of human pancreatic progenitors in the absence of EGF. Human fetal pancreases were dissociated, embedded in Matrigel, and cultured in the complete medium, from which EGF was removed. (A) Both cysts and organoids develop under such conditions and can be passaged. Scale bars: 250 μm. (B) After 4 passages, cell were fixed, embedded in paraffin, sectioned, and stained with anti-PDX1, anti-SOX9, anti-E-cadherin, anti-NKX6-1, anti-NKX2.2, and anti-NGN3 antibodies. Scale bars: 50 μm. (C) After 4 weeks in culture, cell aggregates were dissociated using trypsin and seeded in Matrigel-coated plates for culture in 2D. Five days later, cells were fixed, and stained with anti-SOX9, anti-PDX1, anti-E-cadherin, anti-NKX6-1, anti-chromogranin A (CHGA), anti-insulin, anti-somatostatin (SST), and anti-glucagon (GCG) antibodies. Scale bars: 50 μm.

EGF CONTROLS FETAL PROGENITOR FATE IN VITRO
FIG. 3. EGF is a strong repressor of endocrine differentiation. Quantitative polymerase chain reaction (PCR) showing relative fold change of PDX1, LGR5, SOX9, NKX6-1, SPP1, PTF1A, NGN3, NEUROD, NKX2-2, PAX4, CHGA (chromogranin A), INS (insulin), GCG (glucagon), and SST (somatostatin) mRNA levels in cysts and organoids from human fetal pancreas cultured for 28 days with EGF (black bars) and without EGF (gray bars). Cyclophilin A was used to normalize for RNA input. Data represent mean ± SD (n = 4 from independent human fetal pancreases).
culture, 2 to 10 eGFP\textsuperscript{high}mRFP\textsuperscript{+} cells were detected among 20,000 cells counted and plated, indicating that only very few of the early progenitors were already committed to the beta cell fate (Fig. 4B). Cells amplified in EGF-containing medium gave rise to cyst-like epithelial structures with prevalent ductal phenotype, being a single layer of cells expressing pan-cytokeratin (Supplementary Fig. S5). Moreover, they were Pdx1\textsuperscript{+}, Sox9\textsuperscript{+}, Nkx6.1\textsuperscript{+}, and E-cadherin\textsuperscript{+}, as expected for progenitor cells, and also duct marker, mucin1, but did not express the pan-endocrine marker, synaptophysin (Supplementary Fig. S5).

Ngn3 transcripts were abundant at isolation (E12-E13), but decreased once the cells were plated in Matrigel and expansion medium (Fig. 5). On the contrary, in the absence of EGF, few cystic structures appeared and the number of eGFPhighmRFP\textsuperscript{+} cells increased from day 3 onward (Fig. 4C) and, similar to fetal human pancreatic cells cultured without EGF, they lost their monolayer appearance and became spheres filled with cells (Fig. 4C). At day 3, Ngn3 gene transcription was rapidly and transiently activated, preceding the appearance of mature cells with the endocrine phenotype (not shown). In the absence of EGF, cells could be split 1:2 on a weekly basis and expanded for only 4–5 weeks. Following 7 days in EGF-deprived culture medium, not only the level of NeuroD, Nkx2.2, and Nkx6.1 but also insulin, glucagon, and somatostatin transcripts increased dramatically (10- to 50-fold) and synaptophysin\textsuperscript{+} and insulin\textsuperscript{+} cells appeared (Fig. 5). Similar to humans, fetal pancreas from the mouse thus contains progenitor cells that can be expanded in the long term as duct-like structures in the presence of EGF and differentiated toward endocrine cells in its absence.

To test whether single progenitor cells recapitulate this expansion/differentiation process, independently from neighboring cells, a cell suspension was obtained from pancreases of WT and Pdx1eGFPMipmRFP\textsuperscript{+} mice following collagenase and trypsin digestion. EpCAM was used as the

![FIG. 4. In vitro expansion and differentiation of mouse fetal progenitors derived from Pdx1eGFPMipmRFP. (A) Schematic representation of mouse embryonic epithelial cell isolation from Pdx1eGFPMipmRFP mice. (B) Expansion of epithelial cells derived from Pdx1eGFPMipmRFP mice in the presence of EGF: eGFPM\textsuperscript{+} progenitors progressively downregulate eGFP reporter expression and amplify as duct-like structures. Rare mRFP\textsuperscript{+} cells are detected within the first 3 days of culture, but not at later time points. (C) Expansion of epithelial cells derived from Pdx1eGFPMipmRFP mice in the absence of EGF: eGFPhigh and mRFP\textsuperscript{+} double positive cells progressively appear in culture and organize prevalently as spheres filled with cells. All cultures are over 3 passages (every 7 days) for 28 days, total time in culture. Scale bars: 100 μm.](image)
FIG. 5. Real-time PCR analysis of embryonic mouse progenitors in 3D cultures over 1 month of expansion. Graphs representing relative fold changes for Pdx1, Lgr5, Sox9, Ptf1a, Nkx6.1, Spp1, Ngn3, NeuroD, chromogranin-A (ChrA), Nkx2.2, Pax4, Ins1 (insulin-1), Sst (somatostatin), and Gcg (glucagon) transcript levels of embryonic mouse progenitors in EGF expansion medium (black bars) and without EGF (gray bars) cultured for 28 days. Cyclophilin A was used for normalization of RNA input. Data represent mean ± SEM (n = 3 independent cultures).
FIG. 6. Prospective isolation of mouse fetal progenitors derived from Pdx1^eGFP^Mip^mRFP^ (A) Schematic representation illustrating fetal pancreatic dissociation for fluorescence-activated cell sorting (FACS). (B) FACS panels showing the cell sorting strategy: characteristic profile and gating of live cells [propidium iodide (PI)-negative, left panel], followed by gating of eGFP^EpCAM^ epithelial cells (central panel); representative purity (~100%) of sorted eGFP^EPCAM^ cells that are negative for TSQ (right panel). (C) Graph representing exponential growth of sorted fetal pancreatic progenitors over 28 days in expansion medium. (D, E) Expansion of prospective isolated epithelial cells derived from Pdx1^eGFP^Mip^mRFP^ mice in the presence and in the absence of EGF: EpCAM^eGFP^ progenitors progressively downregulate eGFP reporter expression and amplify as duct-like structures in expansion medium (D), while double positive eGFP^high^mRFP^ progenitors appear in culture when cultivated in the absence of EGF (E). Scale bars: 100 μm. (F) Immunohistochemistry confirms epithelial progenitor marker expression (E-Cad^+, Sox9^+, Nkx6.1^+ cells) and the absence of insulin^+ cells. (G) Immunohistochemistry analysis for endocrine markers demonstrates the presence of monohormonal INS^+, GCG^+, and SST^+ endocrine (SYP^+) cells when cells are cultivated in the absence of EGF. Insets show single hormonal cells at higher magnification: INS co-stains with SYP, but not with SST and GCG. Scale bars: 50 μm.
pan-epithelial cell surface marker, and eventual endocrine cells were identified by their active insulin promoter (detected by mRFP expression) and/or their high degree of granulation (detected by incubating the cells with the fluorescent chelator TSQ that binds to Zn$^{2+}$ in secretory granules [18]), while eGFP expression allowed sorting of pancreatic progenitors (Fig. 6A, B). Single EpCAM$^{+}$TSQ$^{-}$ (WT mice) or EpCAM$^{+}$eGFP$^{+}$mRFP$^{+}$TSQ$^{-}$ (Pdx1$^{+}$GCG$^{+}$Ins$^{+}$mRFP$^{+}$) cell suspensions were purified by fluorescence-activated cell sorting and seeded in 3D Matrigel at different concentrations, including clonal density (Fig. 6C, D, and Supplementary Fig. S6). In all of 10 independent experiments, sorted single cells obtained from WT or reporter mice could be expanded exponentially in the long term as E-cadherin$^{+}$Sox9$^{+}$Nkx6.1$^{+}$ epithelial cells (Fig. 6F). These findings confirm the possibility of prospective isolation of single pancreatic progenitor cells and their responsiveness to Wnt independently from support by other cell types. Importantly and similar to bulk cultures of fetal human pancreatic cells, exclusion of EGF from the medium allowed endocrine differentiation documented by the appearance of eGFP$^{+}$PhighmRFP$^{+}$ cells (Fig. 6E) and confirmed by detection of monohormonal INS$^{+}$SYP$^{+}$, INS$^{+}$GCG$^{+}$, INS$^{+}$STT$^{+}$, INS$^{-}$GCG$^{-}$, and INS$^{-}$STT$^{-}$ cells (Fig. 6G).

Discussion

Our data describe efficient in vitro expansion in 3D cultures of fetal progenitors from both human and mouse pancreases. In addition, we show that EGF behaves as a gatekeeper for endocrine differentiation of fetal pancreatic progenitors when cultured under these conditions. Therefore, these cultures represent a powerful model to study both environmental signals and intrinsic factors that regulate pancreatic progenitor proliferation and differentiation toward mature endocrine cells. Importantly, it offers an in vitro model system suitable to address human beta cell maturation and may contribute to the development of a platform for testing new compounds affecting differentiation of progenitors and/or beta cell function. The possibility to study and manipulate ex vivo both proliferation and differentiation of bona fide human progenitors represents a novel and unique system for understanding human beta cell development.

Our aim was to isolate progenitor cells from the fetal pancreas of both mouse and man at defined stages of development, challenge their growth potential in vitro, and characterize them during culture. We used conditions similar to the ones we have previously developed to expand adult mouse pancreatic progenitors [2]. Cells expanded in long-term culture, expressed progenitor markers, and displayed a ductal phenotype. Importantly, we obtained the same result when single epithelial cells initiated the cultures, demonstrating that progenitor expansion was independent from other cell types. After successful expansion of both fetal mouse and human progenitors, we assessed their differentiation potency by manipulating the culture conditions. Therefore, small cell aggregates were isolated from the pancreas of 8–11 weeks of development for humans and E12 or E13 for mice, stages known to be enriched for progenitors able to give rise to all differentiated lineages of adult pancreas [12,19,20].

Our data strongly support the hypothesis that in vitro beta cells can be generated from fetal pancreatic progenitors. Indeed, when fetal epithelial cells either as small aggregates or as single cells were cultured in the absence of EGF, endocrine progenitor markers, such as NGN3 that plays a central role in endocrine cell differentiation [19,21] and NKX2-2, became detectable. Interestingly, NKX2-2 has recently been reported as important in human endocrine development as mutations of this gene cause neonatal diabetes with preservation of exocrine function [1,22]. Finally, we report that fetal progenitors have the ability to differentiate into monohormonal endocrine cells, including the beta cell that expresses a high level of the transcription factors, PDX1 and NKX6-1. The robustness of our in vitro model is confirmed by the high reproducibility of our experiments with 100% efficiency to expand progenitor cells from human pancreases, independently from their different stage of development and genetic background.

The vast majority of the studies on pancreas development focus on the mouse. However, a direct translation from mouse to man is not obvious. In a previous report, we have shown that ectopic development of human and mouse embryonic pancreases in vivo is similar, despite a much faster development of mouse compared to human [23]. We here compared ex vivo growth of progenitor cells dissociated from fetal pancreas from a human and mouse and demonstrated that both respond to the same environmental signals. More specifically, we disclose a role for EGF on fate choice of early progenitors of the pancreas. These data further validate the mouse as a model system for understanding human pancreatic development.

Moreover, EGF receptor (EGF-R) is expressed by the pancreatic epithelium during all stages of development and by endocrine cells in adult pancreas [24–26]. EGF signaling plays an important role during pancreatic development that is affected in EGFR$^{-/-}$ mice that also show an impaired beta cell mass and disturbed islet migration [25]. In addition, signaling through EGFR seems also important for islet architecture as, in EGFR$^{-/-}$ mice, the endocrine cells are located in streak-like structures associated with the pancreatic duct [25].

We previously reported that EGF stimulates proliferation and inhibits endocrine differentiation in cultured E12.5 rat pancreatic explants [27]. Furthermore, EGF-R is required for normal beta cell development and postnatal beta cell proliferation [28], and administration of EGF to large animals, such as pigs, also induces duct cell proliferation, while only a limited number of insulin$^{+}$ cells develop [29], supporting preservation of this EGF effect among species. Interestingly, in the adult pancreas, EGF induced transdifferentiation of acinar cells to ducts [30–32] and also played a role in acinar to beta cell reprogramming following chronic diabetes in mice [33]. In addition, EGFR signal transduction affects proliferation of pancreatic progenitors and blocks their differentiation from human ES cells [34].

Our data thus support the complex role of EGF on pancreatic development and beta cell maintenance. They offer a novel tool to study human and mouse pancreatic progenitors in vitro under defined conditions and indicate that EGF can control both proliferation and differentiation of early progenitor cells. This model system could be used to study mechanisms that control proliferation and fate decision of
pancreatic progenitors to identify additional signaling pathways and growth factors necessary for endocrine differentiation and beta cell maintenance ex vivo.

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