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Induction of mouse pancreatic ductal differentiation, an in vitro assay

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Abstract Despite recent technical advances for studying lineage tracing and gene functions, our knowledge of pancreatic duct progenitor cells and mechanisms involved in their differentiation remains a huge void in our understanding of pancreatic development. A deeper insight into ductal differentiation is needed because ductal cells may harbor pancreatic stem/progenitor cells that could give rise to new islets. Also, since the most common pancreatic tumors form structures expressing ductal cell-specific markers, studies of ductal development may provide better markers for pancreatic tumor classification. One major longstanding problem in the study of pancreatic ductal differentiation has been the lack of an effective in vitro model. We thus wished to develop an in vitro system for the study of pancreatic duct development. In doing so, we have developed a specific culture condition to promote ductal differentiation of E11.5 pancreatic rudiments. Normally, pancreatic explants cultured in vitro develop to form endocrine, acinar, as well as ductal cells. Here, we report that addition of a combination of EGF, fibroblast growth factor-10, and platelet-derived growth factor-AA to the explant cultures promotes ductal differentiation, while preventing endocrine and acinar differentiation. This culture system for differentiation and enrichment of pancreatic ductal cells may allow identification of gene(s) involved in ductal development.

Keywords Pancreas · Development · Differentiation · Duct · Growth factors · Mouse

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Introduction

The pancreas is an organ of endodermal origin with two major cell populations, endocrine and exocrine cells. The endocrine portion consists of clusters of hormone-producing cells, the islets of Langerhans, which are scattered within the exocrine tissue. The highly branched exocrine compartment, which makes up the majority of the pancreas, is composed of the acinar exocrine secretory cells, mainly producing digestive enzymes, and the excretory ducts, continuous with the digestive tract. Lineage tracing studies have shown that all pancreatic epithelial cells originate from a pool of $PDX1^+$ and PTF1a-$p48^+$ progenitors (Gu et al. 2002; Kawaguchi et al. 2002). These early multipotent progenitor cells also express lineage-selective transcription factors such as SOX9 and HNF1β (Lynn et al. 2007; Solar et al. 2009).

It has been known since the 1960s that soluble factors originating from the mesenchyme play a functional role in

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pancreatic development (Golosow and Grobstein 1962; Wessells and Cohen 1968). These mesenchymal signals are especially essential for differentiation of the acinar cells (Gittes et al. 1996). Several growth factors have been demonstrated to be critical for growth and expansion of the developing pancreas. Fibroblast growth factor-10 (FGF10) is expressed in the pancreatic mesenchyme as early as E9.5 and is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. In Fgf10$^{-/-}$ embryos, after initial evagination of the dorsal and ventral buds, the subsequent growth, differentiation, and branching morphogenesis of the pancreatic epithelium are arrested, possibly due to a dramatic reduction of PDX1 production (Bhushan et al. 2001). FGFR2IIIB, a receptor for FGF10 is expressed in undifferentiated pancreatic epithelial cells until E13.5, while subsequently its expression becomes restricted to the β-cells (Hart et al. 2003). Pancreas in mice lacking FGFR2IIIB is noticeably smaller than the wild-type littermates during embryogenesis, and pancreatic ductal branching as well as duct cell proliferation is significantly reduced. However, both exocrine and endocrine pancreatic differentiation seems to occur normally (Pulkkinen et al. 2003). Furthermore, ectopic expression of FGF10 in pancreatic progenitor cells stimulates proliferation and blocks differentiation as a result of persistent Notch signaling (Hart et al. 2003; Norgaard et al. 2003). Epidermal growth factor receptor (EGFR) and several members of the EGFR family are expressed in the developing pancreas (Kritzik et al. 2000; Huotari et al. 2002). Egfr$^{-/-}$ mice show disturbed migration and delayed differentiation of β-cells in the developing pancreas (Miettinen et al. 2000; Cras-Meneur et al. 2001). In a previous study, we demonstrated that transforming growth factor-alpha, a ligand for EGFR, prevented acinar development in E10.5 pancreatic explant cultures indicating that differentiation of acinar cells may be controlled by EGFR signaling (Esni et al. 2004b). Platelet-derived growth factors (PDGFs) appear to act on specific populations of progenitor cells that generate distinct cell types in a number of developmental processes (Betsholtz et al. 2001). In rats, the expression of PDGF receptors was reported in the E13 pancreas (LeBras et al. 1998). Moreover, it has been shown that in freshly isolated cells from the adult pancreatic ductal network, FGF10, PDGF-AA, and epidermal growth factor (EGF) stimulate phosphorylation of p38 mitogen-activated protein kinase (Kayali et al. 2005). Several studies have demonstrated that although FGF10, FGFR2IIIB, and EGFR control branching of the exocrine pancreas, the effect is mainly on the proliferation of cells rather than on differentiation (Miettinen et al. 2000; Bhushan et al. 2001; Cras-Meneur et al. 2001; Pulkkinen et al. 2003).

A better understanding of ductal differentiation is badly needed because ductal cells may harbor pancreatic stem/progenitor cells that could give rise to new islets. It is widely accepted that under physiological conditions, β-cell regeneration in the adult mouse pancreas typically originates from β-cell self-duplication (Dor et al. 2004; Teta et al. 2007). However, depending on the type of injury model, it appears that new β-cells can arise from cells residing within the ducts (Bonner-Weir et al. 2008; Inada et al. 2008; Criscimanna et al. 2011), in proximity to the ductal network (Xu et al. 2008), or even from α-cells (Chung et al. 2010; Thorel et al. 2010; Yang et al. 2011).

The mature pancreatic ductal compartment consists of the main duct, interlobular ducts, intralobular ducts, and finally the intercalated ducts, which drain the acinar cells. Little information is available regarding the developmental timing and programs controlling pancreatic ductal differentiation and growth. Lineage tracing studies indicate that ductal progenitor cells specifically express PDX1 between E9.5 and 11.5 (Gu et al. 2003). Cells expressing PDX1 before or after these stages will differentiate either to endocrine or acinar cells, which would suggest that selection of progenitor cells between endocrine/acinar and ductal lineages has occurred by E12.5. Analysis of *Hnf-6*$^{-/-}$ pancreas has indicated requirement of HNF-6 for normal development of the inter- and intralobular segments of the pancreatic duct (Pierreux et al. 2006). The lack of specific markers has probably been the major obstacle to studying ductal development in the pancreas. Cytokeratins (CK-7, 8, 9, 18, 19, and 20) are often used as duct markers and are found as early as E12.5 in mice (Pulkkinen et al. 2003). However, cytokeratins along with the other available duct markers such as mucins (Muc1 and 5), carbonic anhydrase II, cystic fibrosis transmembrane conductance regulator, and osteopontin are often co-expressed at lower level in endocrine or acinar cells (Grapin-Botton 2005; Kilic et al. 2006; Inada et al. 2008). Lectins such as *Dolichos biflorus* agglutinin (DBA) have also been used to detect the pancreatic duct cells (Kobayashi et al. 2002). Although, DBA binds to differentiated duct cells in the embryonic and adult pancreas, it also binds to undifferentiated "duct-like structures" during pancreatic development. These duct-like structures also harbor progenitors for pancreatic cell types other than duct cells (Gu et al. 2002; Solar et al. 2009). Similarly, transcription factors such as Hes1, Sox9, and Hnf1β which are expressed in duct cells (and/or in the centroacinar cells) in the adult pancreas are also found in the duct-like structures during pancreatic development (Seymour et al. 2007; Solar et al. 2009; Kopinke et al. 2011; Kopp et al. 2011; Shih et al. 2012).

To confront the issue of the lack of an effective in vitro model to study pancreatic ductal differentiation, we have developed a specific culture condition to promote the differentiation of this cell type within E11.5 pancreatic rudiments. This culture system may be used in future studies to better understand the mechanism behind pancreatic ductal differentiation.

Material and Methods

Mice. Mice used in these studies were maintained according to protocols approved by the University of Pittsburgh IACUC.

In vitro explant culture. Explants obtained from E11.5 dorsal pancreatic rudiments were cultured as described previously (Esni et al. 2005). Explants were cultured in pancreatic culture medium (medium-199, complemented with 50 U/ml G-streptomycin, and 1.25 μg/ml Fungizone®, in addition to different serum concentrations).

Whole-mount immunostaining. For whole-mount immunostainings, tissues were fixed and stained as previously described (Esni et al. 2001).

Immunostaining. The following antibodies were used at the indicated dilutions for immunofluorescence analysis: goat anti-amylase 1:500 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rat anti-E-cadherin 1:200 (Zymed, Carlsbad, CA); rabbit anti-cytokeratin 1:100 (DAKO, Carpinteria, CA); Armenian hamster anti-MUC-1 1:100 (Neomarkers, Fremont, CA); and DBA fluorescein isothiocyanate (FITC)- or rhodamine-conjugated 1:100 (Vector Laboratories, Burlingame, CA). The following reagents were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA): Cy2- and Cy3-conjugated donkey anti-rabbit 1:300; donkey anti-rat 1:300; and FITC anti-Armenian hamster 1:100. Images were collected on a Zeiss Imager Z1 microscope with a Zeiss AxioCam driven by Zeiss AxioVision Rel. 4.7 software.

Quantitative RT-PCR. Messenger RNA isolation and subsequent complementary DNA synthesis were performed using μMACS® One-step cDNA Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. PCR primers were purchased from Qiagen (QuantiTect® Primer Assays, Qiagen, Valencia, CA) and are listed in Supplementary Table 1. Reactions were performed with PerfeCTa SYBR Green SuperMix for IQ (Quanta BioSciences, Gaithersburg, MD, cat. 95053) using an IQ5 instrument (Bio-Rad, Berkeley, CA). Reactions were performed in triplicates. Specificity of the amplified products was determined by melting peak analysis. Quantification for each gene of interest was performed with the $2^{-[\mathrm{delta}][\mathrm{delta}]\mathrm{Ct}}$ method. Quantified values were normalized against the housekeeping gene GAPDH, which proved to be stable across the samples.

Results

Ductal culture system development. In a previous study, Kayali et al. reported a proliferative responsiveness by adult pancreatic ductal epithelial cells to the addition of 10 ng/ml EGF, 50 ng/ml FGF10, and 10 ng/ml PDGF-AA (Kayali et al. 2005). To determine whether the embryonic pancreatic cells could show similar responsiveness, we studied the effect of these growth factors on pancreatic ductal differentiation in embryonic pancreatic organ culture. To do so, E11.5 dorsal pancreatic buds were dissected and cultured for 6 d with or without added exogenous EGF (10 ng/ml), FGF10 (50 ng/ml), and/or PDGF-AA (10 ng/ml). In an effort to find the optimal conditions for ductal differentiation, we altered one at a time the serum concentrations in the media (0, 1, and 10%), the specific combination of added growth factors, the substrates on which explants were cultured (membrane, soft agar, and plastic), and also the embryonic tissues used (intact bud or naked epithelium) were altered one at a time. First, we cultured the explants in pancreatic culture medium (PCM) (see "Material and Methods" for details) containing 1% serum supplemented with one of eight possible different combinations of the growth factors. These growth factor combinations were used to grow the explants in soft agar, directly on the bottom of 24-well plates, or on top of Millicell-CM inserts, for a total of 24 different conditions. Subsequently, we cultured either intact (epithelium with mesenchyme) or naked (epithelium without mesenchyme) E11.5 dorsal pancreatic buds in medium containing 1% serum, resulting in a total of 48 different conditions. Following these experiments, we then cultured the explants in medium supplemented with 0 or 10% serum. All together, we thus tested 144 different conditions, and in order to check for reproducibility, each culture condition was repeated five times. Explants were cultured for 6 d while being monitored daily and assessed for the presence of duct-like structures (Fig. 1). After analyzing multiple conditions, it appeared that the ideal conditions for our unique ductal differentiation model were when intact E11.5 dorsal buds were cultured in the presence of 10 ng/ml EGF, 50 ng/ml FGF10, and 10 ng/ml PDGF-AA in PCM containing 1% heat-inactivated fetal calf serum. Differentiation into duct cells was independent of the substrate; however, for practical reasons, it was easier to monitor and compare the growing explants on a daily basis when explants were cultured on plastic. The data shown here represent the intact E11.5 dorsal bud explants cultured in medium containing 1% serum on plastic with (EFP) or without (control) added exogenous growth factors.

Expansion of duct-like structures at the expense of acinar cell differentiation. Normally, pancreatic explants that are cultured in vitro form endocrine, acinar, and ductal components (Esni et al. 2004a, b, 2005). While it is difficult to detect endocrine cells by light microscopy, the zymogen granules in the acinar cells and the morphology of the ducts and duct-like structures make these cell types fairly easy to

discern. Interestingly, morphological differences between explants cultured with growth factors and those cultured without growth factors were apparent as early as day 1 of culture (Fig. 1). In control explants, epithelial branching proceeded normally, and between days 4 and 5, acinar cells were filled with opaque zymogen granules (Fig. 1*A*). However, zymogen granules were seldom detected in explants cultured with the growth factors, and instead, these explants developed a massive network of tubular structures and, in some cases, cysts (Fig. 1*B*). To evaluate the epithelial nature of these tubular structures, whole-mount immunofluorescent analyses using antibodies against the epithelial marker E-cadherin were performed on day 6 explants. As demonstrated in Supplementary Fig. 1, E-cadherin expression in these tubular and cystic structures verified their epithelial character (Fig. S1). Furthermore, these tubular structures uniformly expressed cytokeratin (Fig. 1*B*). To study acinar differentiation, the explants were stained for amylase, a marker for terminally differentiated acinar cells. As demonstrated in Fig. 2, in the absence of exogenous growth factors, both E-cadherin$^+$/amylase$^-$ trunks (arrows in Fig. 2*C*) and E-cadherin$^+$/amylase$^+$ branching tips (arrowheads in Fig. 2*C*) had formed. On the other hand, explants supplemented with growth factors consisted mainly of E-cadherin$^+$/amylase$^-$ epithelium, and the number of E-cadherin$^+$/amylase$^+$ branching tips was greatly reduced (Fig. 2*B*, *D*). The absence of amylase-producing cells was accompanied with a significant reduction in *Ptf1a*, *Mist1*, *Cpa*, and *amylase* gene expression in day 6 EFP-treated explants (Fig. 2*E*). Muc1 is a membrane protein expressed in the ductal network both during development and in the adult pancreas (Cano et al. 2004; Kopinke and Murtaugh 2010). Notably, concomitant with the absence of mature acinar cells, we observed an increase in the relative number of MUC1-positive structures compared to the overall number of epithelial cells in EFP-treated explants (Fig. S2). These data suggest that under culture conditions described above, the putative pancreatic ductal differentiation is promoted at the expense of acinar cell differentiation.

Onset of ductal differentiation. Embryonic pancreas when cultured in vitro forms both endocrine and exocrine cells; however, due to the stress that they are subjected to during dissection, the explants tend to stay behind in development for 1 d when compared to developing pancreas in utero. Thus, day 2, day 4, and day 6 explants (dissected on E11.5 and set as day 0) should be comparable to E12.5, E14.5, and E16.5 pancreas, respectively. MUC1 and DBA staining further confirmed the similarities in ductal development between the control explants

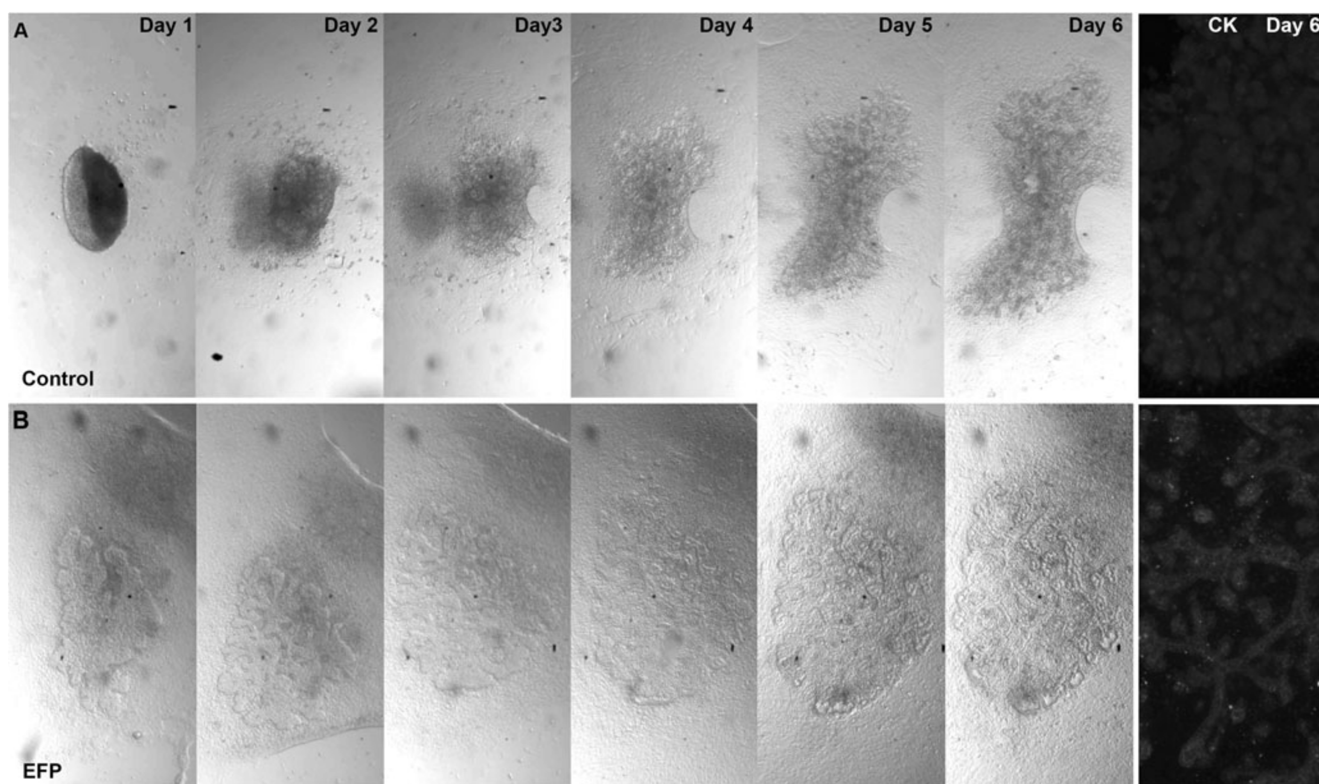

Figure 1. Culture monitoring of mouse pancreatic explants. Day by day growth of intact E11.5 dorsal pancreatic bud explants in the absence (*A*) or presence (*B*) of EFG, FGF10, and PDGF-AA. Morphological differences between the explants cultured with or without supplemented growth factors are evident as early as on day 1. The cells within ductal network on day 6 are cytokeratin-positive.

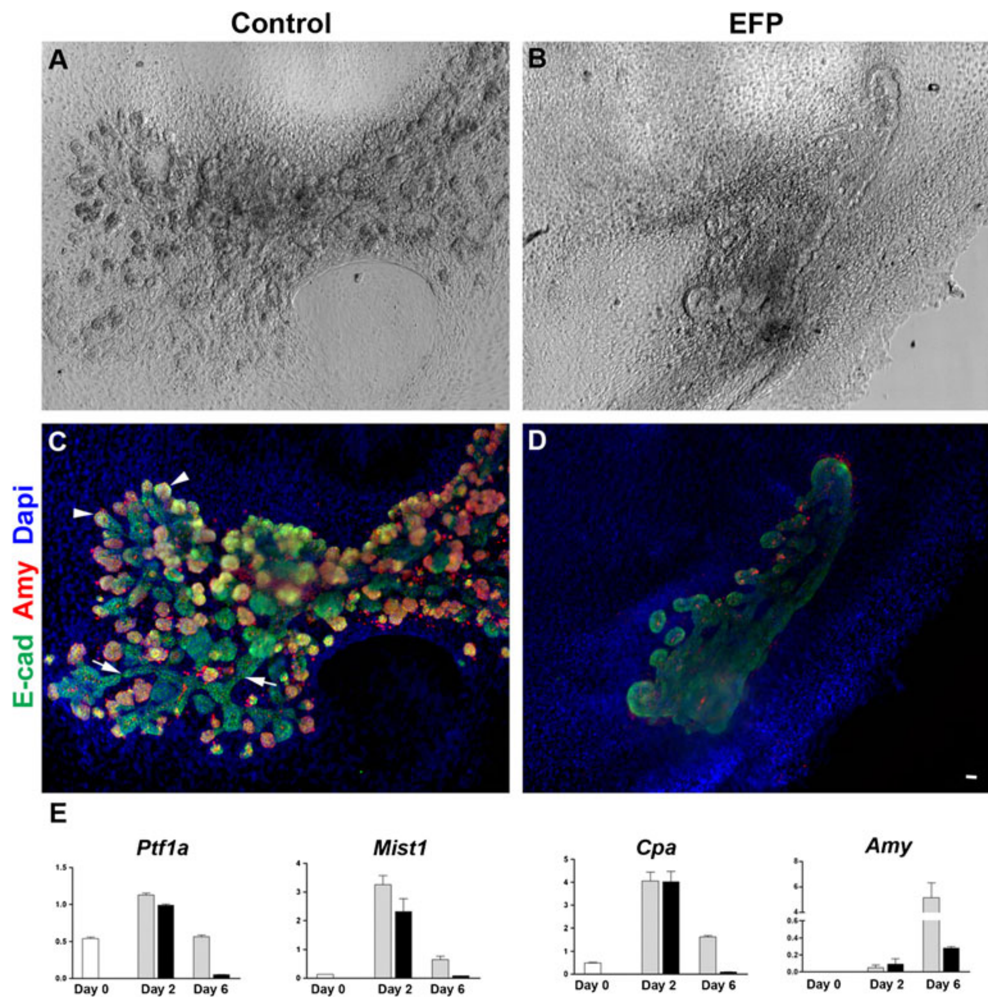

Figure 2. Inhibition of acinar cell differentiation in growth factor-treated explants. Phase-contrast images of control (*A*) and EFP-treated (*B*) explants after 6 d in culture. Whole-mount double immunofluorescent detection of E-cadherin and amylase in control explants (*C*) and explants treated with growth factors (*D*) shows impaired acinar differentiation as the result of added growth factors. The *arrows* in (*C*) mark an epithelial trunk, whereas the *arrowheads* highlight branching tips. qRT-PCR analyses for acinar markers on RNA extracted from E11.5 intact dorsal pancreas (*white bars*) or explants cultured in the presence (*black bars*) or without (*gray bars*) growth factors and harvested on day 2 or day 6. *Scale bar* 20 μm.

and the corresponding embryonic stage in the developing pancreas (compare Figs. 3 and S2 with Fig. S3A, C).

Following optimization of the conditions for ductal differentiation, the next step was to determine the time point at which ductal differentiation was induced. To do so, the explants were harvested at 24-h intervals during the culture period and stained with DBA. As shown in Fig. 3, the number of DBA-positive structures on day 2 was similar in explants cultured with (Fig. 3*B*) or without (Fig. 3*A*) the growth factors. However, starting on day 4, explants treated with EFP (Fig. 3*D*) showed a significant increase in the number of DBA-positive cells when compared to the control explants (Fig. 3*C*). The difference in the number of DBA-positive structures became even more evident on day 6 (Fig. 3*E, F*), when the vast majority of epithelial cells in EFP-treated explants were also DBA-positive. These results indicate that ductal differentiation as the result of supplemented exogenous growth factor in this culture system is initiated around day 4.

Inhibition of endocrine cell differentiation. DBA and Muc1 are both detected in mature ductal cells; however, during pancreatic development and prior to ductal differentiation, these markers are also detected in undifferentiated duct-like structures (Kobayashi et al. 2002; Kopinke and Murtaugh 2010). In order to determine whether the increased number of cells positive for DBA and MUC1 in EFP-treated explants (Figs. 3 and S2) represented undifferentiated duct-like cells or true differentiated duct cells, we compared the amount of mRNA for markers known to be expressed different pancreatic cell types in EFP-treated and control explants on days 0 (E11.5 intact dorsal bud), 2, and 6 of culturing period (Figs. 2*E* and 4). Explants cultured under both conditions harvested on day 2 showed similar expression profile for *Hes1*, *Pdx1*, *Ptf1a*, and also for genes which are expressed in acinar (*Mist1*, *Cpa*, and amylase) or the endocrine (*Ngn3*, *Pax6*, *MafA*, *MafB*, *insulin*, and *glucagon*) lineages. Surprisingly, on day 2, the expression of *Sox9*, *Hnf1β*, *CA-II*, and *Ck19*, which are known to be duct cell markers, was lower in the EFP-treated explants. On day 6, both acinar- and endocrine-specific markers (except for *MafB*) were expressed significantly higher in the control explants. Among duct markers, cytokeratin-19 displayed higher expression levels in the EFP-treated explants, whereas *Sox9*, *Hnf1β*, and *CA-II* were expressed at similar levels in both groups.

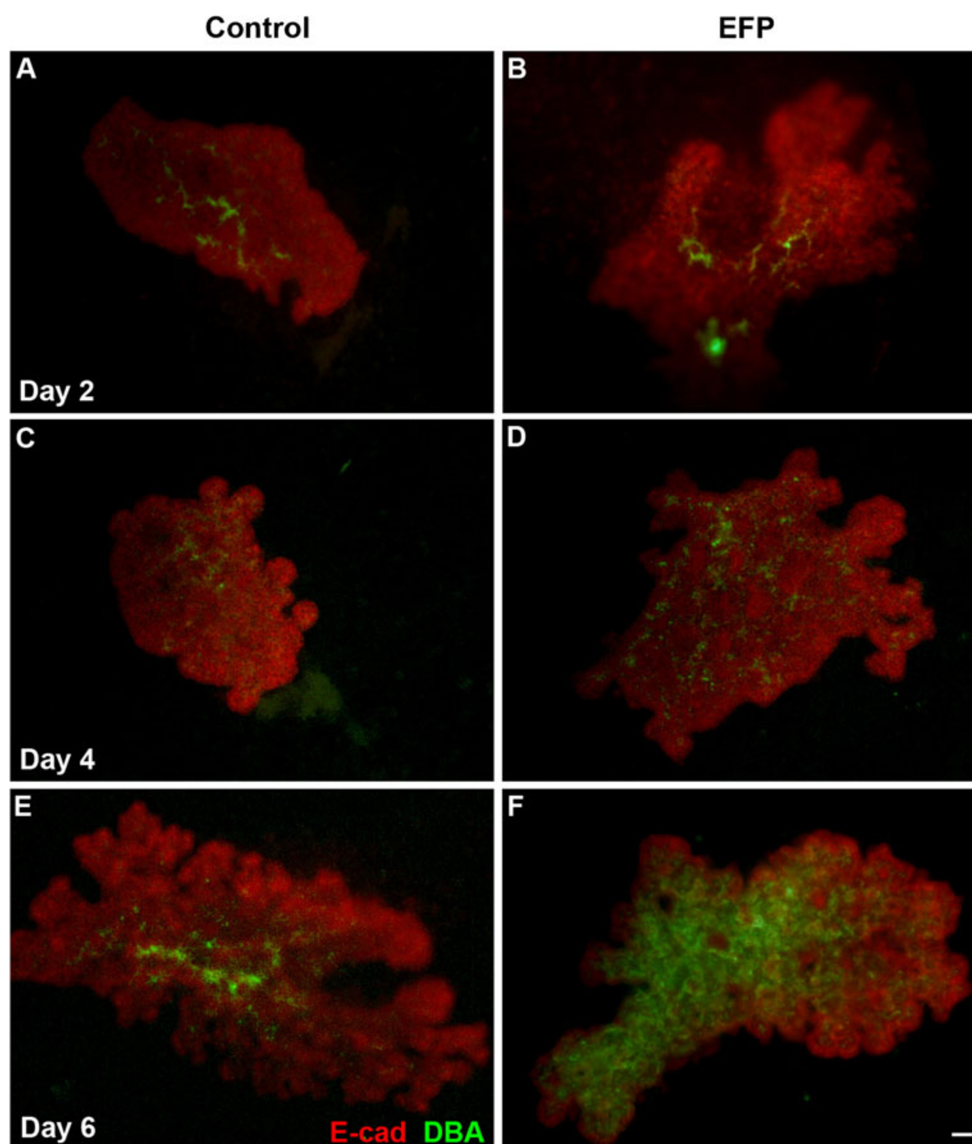

Figure 3. Induced ductal differentiation in growth factor-treated explants. Whole-mount double immunofluorescent analyses of day 2 (*A*, *B*), day 4 (*C*, *D*), and day 6 (*E*, *F*) explants cultured in the absence (*A*, *C*, *E*) or presence (*B*, *D*, *F*) of added growth factors using FITC-conjugated DBA and antibodies against E-cadherin. Starting on day 4, explants treated with EFP (*D*) showed a significant increase in the number of DBA-positive cells when compared to the control explants (*C*). *Scale bar* 20 μm.

These data suggest that under culture conditions described above, pancreatic ductal differentiation is promoted, whereas endocrine cell differentiation is inhibited.

Discussion

Although many genes and pathways have been demonstrated to play functional roles in the developing pancreas, our understanding of pancreatic ductal differentiation is still very limited. In particular, the identification of transcriptional regulators expressed specifically in the developing or differentiated pancreatic duct cells remains unclear. One major problem in the study of pancreatic ductal differentiation has been the lack of a good in vitro model system. In an attempt to enrich for ductal cells, we have developed a unique in vitro system to specifically promote ductal differentiation of E11.5 pancreatic rudiments. To do so, numerous conditions such as combination of exogenous growth factors, serum concentrations, the substrates on which explants were cultured, and also the embryonic tissues used were tested. Our analyses showed that the ideal conditions for our unique ductal differentiation model were when the intact E11.5 dorsal buds were cultured in the presence of 10 ng/ml EGF, 50 ng/ml FGF10, and 10 ng/ml PDGF-AA in pancreatic culture medium containing 1% heat-inactivated fetal calf serum. The growth and morphological changes could be then easily monitored on daily basis by culturing the explants directly on plastic.

In our duct culture system, ductal development seems to be mainly at the expense of acinar development, consistent with the proposed model by Gu et al., in which ducts and acini have a common progenitor, but then diverge around E12.5 (Gu et al. 2002). As mentioned earlier, day 2 and day 4 explants (dissected on E11.5 and set as day 0) are comparable to

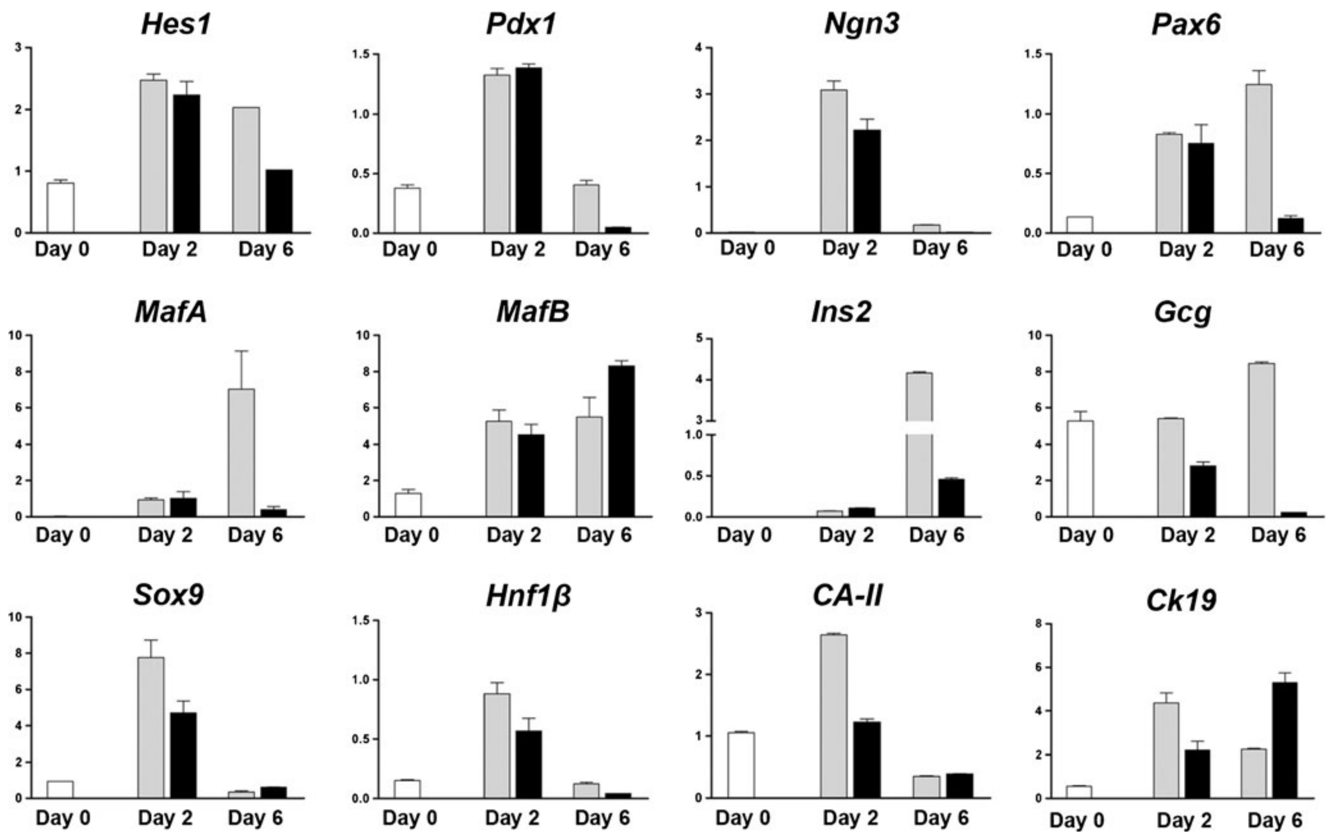

Figure 4. Inhibition of endocrine cell differentiation in growth factor-treated explants. qRT-PCR analysis on RNA extracted from E11.5 intact dorsal pancreas (*white bars*) or explants cultured with (*black bars*) or without (*gray bars*) growth factors and harvested on day 2 or day 6 shows that endocrine markers are dowregulated in EFP-treated explants, while ductal markers are upregulated.

E12.5 and E14.5 pancreas, respectively. Notably, E14 coincides with the start of the secondary transition in pancreatic organogenesis, which is the stage when insulin and amylase begin to be detected (Pictet et al. 1972). Interestingly, although the morphological differences between control and EFP-treated explants could be detected as early as day 1, significant differences could not be observed in the number of cells positive for DBA until day 4 of culture.? Recent findings suggest that separation between the ductal and acinar lineages occurs around E13.5 (corresponding to day 3 in our culture system) (Solar et al. 2009), and that the following expansion of acinar cells depends on their proliferation rather than continues influx of progenitors from duct cells (Zhou et al. 2007). Thus, one explanation for the observed delay could be that the early morphological differences seen on day 1 were due to expansion of the pool of committed but not yet differentiated duct cells, whereas the differences seen from day 4 onward were due to expansion of the ductal cells. In other words, in this culture system and prior to day 4, as a result of added growth factors, progenitor cells normally allocated to acinar and endocrine lineages could become committed duct cell progenitors, while around day 4, the newly differentiated duct cells would start to expand. However, this hypothesis is not consistent with our expression analyses data, where the EFP-treated explants harvested on day 2 displayed the same expression profile for endocrine markers as the control explants. In fact, our results indicate that in the presence of growth factors, the early endocrine commitment occurs, but the subsequent lineage specification is inhibited. The nearly lack of amylase-producing cells along with significant reduction in *Ptf1a*, *Mist1*, and *Cpa* expression on day 6 would imply that acinar differentiation is repressed. In the developing pancreas, these markers are first expressed in undifferentiated epithelial cells, but concomitant with acinar differentiation, their expression becomes gradually restricted to the acinar cells (Kawaguchi et al. 2002; Zhou et al. 2007). Since ductal progenitors are likely to express *Ptf1a* and *Cpa*, our data suggest that although prior to day 4 the total number of undifferentiated cells is the same in both conditions (based on equal *Ptf1a*, *Mist1*, and *Cpa* expression), in EFP-treated explants, the balance between committed ductal and acinar progenitors might have been shifted towards the ductal lineage. In contrast to acinar and endocrine markers, duct markers were either equally expressed in both groups or was upregulated (Ck-19) in growth factor-treated explants. As described previously, the undifferentiated pancreatic duct-like cells express duct markers such as Sox9, Hnf1β, CA-II, and Ck-19, but they also express Hes1, Pdx1, and Ptf1a. The presence of the duct

markers along with the reduced Hes1, Pdx1, and Ptf1a expression would imply that the combined growth factor treatment promotes ductal differentiation.

All together, this culture system for differentiation and enrichment of pancreatic ductal cells may guide us towards a better understanding of the possible mechanisms involved in duct formation and lay the groundwork for a more rigorous analysis in the future. For example, how long could these duct cells be maintained in culture? Additionally, to what degree do explants cultured under described conditions mimic endogenous duct formation?

Conclusion

In summary, we have shown that the addition of EGF, FGF10, and PDGF-AA to explant cultures of E11.5 intact dorsal buds appears to promote ductal differentiation and prevents endocrine as well as acinar differentiation. This culture system for differentiation and enrichment of pancreatic ductal cells may allow identification of gene(s) involved in ductal development.

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