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Epithelial: Endothelial cross-talk regulates exocrine differentiation in developing pancreas

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ABSTRACT

Endothelial cells are required to initiate pancreas development from the endoderm. They also control the function of endocrine islets after birth. Here we investigate in developing pancreas how the endothelial cells become organized during branching morphogenesis and how their development affects pancreatic cell differentiation. We show that endothelial cells closely surround the epithelial bud at the onset of pancreas morphogenesis. During branching morphogenesis, the endothelial cells become preferentially located near the central (trunk) epithelial cells and remain at a distance from the branch tips where acinar cells differentiate. This correlates with predominant expression of the angiogenic factor vascular endothelial growth factor-A (VEGF-A) in trunk cells. In vivo ablation of VEGF-A expression by pancreas-specific inactivation of floxed *Vegfa* alleles results in reduced endothelial development and in excessive acinar differentiation. On the contrary, acinar differentiation is repressed when endothelial cells are recruited around tip cells that overexpress VEGF-A. Treatment of embryonic day 12.5 explants with VEGF-A or with VEGF receptor antagonists confirms that acinar development is tightly controlled by endothelial cells. We also provide evidence that endothelial cells repress the expression of Ptf1a, a transcription factor essential for acinar differentiation, and stimulate the expression of Hey-1 and Hey-2, two repressors of Ptf1a activity. In explants, we provide evidence that VEGF-A signaling is required, but not sufficient, to induce endocrine differentiation. In conclusion, our data suggest that, in developing pancreas, epithelial production of VEGF-A determines the spatial organization of endothelial cells which, in turn, limit acinar differentiation of the epithelium.

Introduction

Pancreas development in the mouse starts with the formation of tissue buds from the ventral and dorsal midgut endoderm. At this initial stage, the pancreas contains epithelial progenitor cells that will give rise to exocrine, endocrine and ductal cells, according to a developmental program tightly regulated by cell autonomous factors and by interactions with the mesenchyme (Cano et al., 2007; Gittes, 2009; Jorgensen et al., 2007; Murtaugh, 2007). At the onset of pancreas morphogenesis, *i.e.* around embryonic day (e) e10.5–e11.5, the epithelial progenitors are organized as a compact mass of non-polarized cells. Microlumina then appear and coalesce to generate the lumen of a branched network of ducts, lined by a single-layered polarized epithelium (Hick et al., 2009; Kesavan et al., 2009; Puri and Hebrok, 2007).

The epithelial cells located at the tip of the branched ducts initially consist of multipotent progenitors. These "tip cells" are characterized by the expression of the transcription factors pancreatic and duodenal homeobox factor-1 (Pdx1), pancreas-specific transcription factor subunit 1a (Ptf1a)/p48 and c-Myc and of the enzyme carboxypeptidase A (CPA) (Zhou et al., 2007). The tip cells divide and give rise, on one hand, to epithelial outgrowths that differentiate to exocrine acini, and, on the other hand, to the trunks of the branched ducts, from which endocrine and duct cells differentiate. Exocrine differentiation is dependent on the transcription factor Ptf1a, which associates with recombining binding protein suppressor of hairless (RBP)-J and RBP-JL and stimulates transcription of acinar-specific genes. The latter include amylase, which serves as a marker that distinguishes tip cells

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from acinar cells, and exocrine differentiation and proliferation factor (Exdpf), which in Zebrafish is essential for exocrine development (Beres et al., 2006; Cockell et al., 1989; Fujikura et al., 2007; Jiang et al., 2008; Krapp et al., 1998; Masui et al., 2007). Mist1, another basic helix–loop–helix factor, is required for acinar maturation and control of exocrine cell proliferation (Jia et al., 2008; Pin et al., 2001; Rukstalis et al., 2003). There is evidence for a role of the Wnt/β-catenin pathway in exocrine differentiation (Murtaugh et al., 2005), and several studies addressing the function of this pathway underscored the role of β-catenin in acinar cell proliferation, mostly by stimulating expression of Myc, a factor required for exocrine development (Bonal et al., 2009; Dessimoz et al., 2005; Heiser et al., 2006; Nakhai et al., 2008; Papadopoulou and Edlund, 2005 ; Strom et al., 2007; Wells et al., 2007). Whereas homolog of hairy/enhancer of Split-1 (Hes-1), an effector of Notch signaling, is not required for exocrine differentiation, sustained Notch signaling activity in the pancreatic epithelium prevents exocrine differentiation (Hald et al., 2003; Jensen et al., 2000; Murtaugh et al., 2003). Indeed, the Notch target genes (Hes-1, Hey-1 and Hey-2) repress Ptf1a activity, independently of changes in the levels of Ptf1a component proteins (Esni et al., 2004). Moreover, activation of the Notch pathway is necessary in endocrine progenitors to sequester RBP-J so as to avoid differentiation of endocrine progenitors into acinar cells (Cras-Meneur et al., 2009). Conversely, the pancreatic mesenchyme promotes exocrine differentiation at the expense of endocrine development (Crisera et al., 2000; Duvillie et al., 2006; Li et al., 2004; Miralles et al., 1998).

Endothelial cells were shown to play a role at an early stage of pancreas development. Around e8.75–e9.5 in the mouse embryo, the aorta and vitelline veins are located near the dorsal and ventral pancreatic buds, respectively. In vitro recombination of endoderm and dorsal aorta from e8.25 to e8.5 mouse embryos induces expression of Pdx-1 and insulin in the endoderm (Lammert et al., 2001). Moreover, in vivo, aortic endothelial cells are required for maintenance of Pdx1 expression, for induction of Ptf1a and for dorsal pancreas budding (Yoshitomi and Zaret, 2004). This further implies a relay pathway in which endothelial cells promote survival of the dorsal pancreatic mesenchymal cells, the latter being a source of secreted factors, such as fibroblast growth factor (FGF)-10, that are required for maintenance of Ptf1a expression in the dorsal pancreatic bud (Jacquemin et al., 2006). Tissue recombination experiments demonstrate that the endothelial cells can control early pancreas development irrespective of the presence of blood flow. However, data suggest that sphingosine 1-phosphate, a blood-borne factor, stimulates pancreatic mesenchyme proliferation and so promotes pancreas budding from the endoderm (Edsbagge et al., 2005).

Beyond the stage of pancreas specification and tissue budding from the endoderm, endothelial cells keep an important function in pancreas differentiation and morphogenesis. Xenopus embryos in which the aorta has been removed fail to express proendocrine genes and insulin (Lammert et al., 2001). In the mouse, overexpression of vascular endothelial growth factor-A (VEGF-A) under control of a Pdx1 promoter induces hypervascularization in the pancreas. At 2 months of age, this is associated with hyperplasia of the endocrine islets, and with reduced acini area, suggesting that vascularization can promote endocrine development at the expense of acinar cells (Lammert et al., 2001). In the postnatal period, endothelial cells and endocrine cells mutually support each other: endocrine cells secrete VEGF-A and endothelial cells produce laminins, which stimulate insulin gene expression and beta cell proliferation (Lammert et al., 2003; Nikolova et al., 2006).

It is presently unknown how blood vessels develop in the pancreas and if blood vessel morphogenesis impacts on the initiation of pancreatic differentiation. To address these questions, we first provided a detailed description of blood vessel localization in the pancreas. We further studied pancreatic explants and transgenic mouse models in which endothelial cell development is either stimulated or inhibited by modulation of VEGF-A signaling. Our results show that VEGF-A expression dictates endothelial cell localization near epithelial trunk cells, and that endothelial development limits acinar differentiation.

Materials and methods

Plasmid

Ptf1a5'enh/Ela1p-Vegfa, used to generate transgenic embryos, was obtained by replacing the elastase promoter and lacZ gene from Ptf1a5'enh/Ela1p-nlslacZ (gift from R. MacDonald) (Masui et al., 2008) by an elastase promoter casette (EcoRV/ClaI) and the Vegfa insert (ClaI/NheI).

Animals

Vegfa-floxed mice were obtained from N. Ferrara (Genentech) (Gerber et al., 1999), Pdx1-green fluorescent protein (Pdx1-GFP) and Pdx1-Cre from D. Melton (Gu et al., 2002). Transgenic embryos were generated as described (Poll et al., 2006), with 5 ng/μl of purified *Ptf1a/ela1p-VEGF-A* fragment (*Xba*I–*Pac*I). All other mice were of the CD1 strain. The animals were raised and treated according to the principles of laboratory animal care of the University Animal Welfare Committee.

Dissection and culture of pancreatic explants

Pancreatic explants were microdissected from e12.5 mouse embryos and cultured on microporous membranes on M199 medium supplemented with 10% fetal calf serum (van Eyll et al., 2004). The medium was changed every day. Recombinant mouse $VEGF_{164}$ (R&D systems, Lille, France) was dissolved to 50 μg/ml (stock solution) in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and added at the final concentration of 150 to 500 ng/ml. SU5416 (VEGFR2 kinase inhibitor III, Calbiochem/VWR, Leuven, Belgium) was dissolved in dimethyl sulfoxide to 10 mM (stock solution) and was added at 5 μM in the culture medium. Oxindole I (Calbiochem/VWR, Leuven, Belgium) was dissolved in DMSO to 50 mM (stock solution) and added at 2 μM in the culture medium. Control explants were exposed to the same concentration of vehicle as the test samples.

In situ hybridization

Mouse embryos were fixed overnight at 4 °C in 60% ethanol, 30% formaldehyde and 10% acetic acid before paraffin embedding. Digoxigenin-labeled antisense RNA probes for Vegfa and VEGF Receptor type 2/Fetal liver kinase 1 (Vegfr2/Flk1), were produced by in vitro transcription of the Vegfa and Vegfr2 cDNAs. Specifically, probes spanning nucleotides 94 to 429 of the mouse coding sequence for VEGF and nucleotides 3361 to 4440 of the mouse coding sequence for VEGFR2 were used. In situ hybridizations were performed on 16-μm-thick sections as described (Jacquemin et al., 2003). Images were acquired using a Zeiss Mirax Midi fluorescence microscope.

Immunolabeling and quantification

For whole-mount immunolocalization pancreata dissected from embryos were fixed in 4% formaldehyde in PBS for 2 h at 4 °C and treated as described (Pierreux et al., 2006). For immunohistochemistry after *in situ* hybridization, sections were treated as described (Gaide Chevronnay et al., 2008). For immunofluorescence on sections, embryos or explants were fixed, embedded and processed as described (Pierreux et al., 2006). Antibodies and dilutions used in this study are described in Supplementary Table 1. The TSA

amplification kit was used to detect CPA. Sections were stained with *bis*-benzimide (Sigma, Bornem, Belgium) or with TOPRO in PBS during incubation with the secondary antibodies. Fluorescence was observed with a Zeiss Axiovert 200 inverted fluorescence microscope, with a Biorad 1024 confocal microscope or with a Zeiss LSM510 multiphoton confocal microscope. Three-dimensional projections were obtained with the LSM510 software. For quantification of Ptf1a-, Paired Homeobox factor-6 (Pax6)- or Neurogenin-3 (Ngn3)-expressing cells, the number of positive cells on every sixth sections of the whole explants was counted. Approximately twenty-five 7-μm-sections, each separated by 42 μm, were analysed. Integration of these numbers per distance interval was performed, and their sum gave an estimation of the total number of cells per explant. Normalization to 100 μm was necessary due to explant size variations. For quantification of endothelial and acinar cell area on sections, the regions expressing platelet cell adhesion molecule (PECAM), E-cadherin (E-cadh) and CPA were selected and scanned with a Zeiss Mirax Midi fluorescence microscope. Images were transferred and analysed with the Zeiss Axiovision Rel 4.7 program, as follows: the pancreas, or a particular region within the pancreas, was delineated on the image and the percentage of the surface occupied by the blue, green or red signal in the selected region was quantified.

Cell sorting

Epithelial and non-epithelial cells from Pdx1-GFP pancreata (e14.5) were sorted based on their fluorescence. GFP/GFP males were mated with CD1 females and 25 GFP-expressing pancreata were microdissected at e14.5. Tissue dissociation was performed in 500 μl of trypsin 0.05% (Gibco) at 37 °C for 10 minutes, with pipet mix every 2 minutes. Reaction was stopped with 500 μl of RPMI + 10% Serum and cellular clumps were removed by passing the cell suspension on a 40-μm nylon filter (Cell Strainer, BD Falcon). The filter was washed once with 1 m of RPMI and the single-cell suspension (2 ml) was sorted on a FACSVentage (Becton Dickinson).

Real-time RT-PCR

Total RNA was extracted from cultured pancreatic tissues or from FACS-sorted cells using Trizol reagents (Invitrogen, Carlsbad, CA). RNA (0.5–1 μg) was reverse-transcribed with random hexamers using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed in duplicate by using SYBR Green Supermix (Invitrogen, Carlsbad, CA). Primers sequences are described in Supplementary Table 2. The relative changes in target gene/β-actin mRNA ratio were determined by transformation of threshold cycles to absolute mRNA numbers (Pierreux et al., 2006).

Results

A network of blood vessels embraces the developing pancreatic epithelium

To investigate the role of blood vessels in pancreas development, we first determined their spatial organization in relation to the pancreatic epithelium (Fig. 1). At e11.5, when the pancreas consists of a mass of epithelial cells (labeled for E-cadherin expression), endothelial cells (detected by PECAM staining) formed an irregular network all around the tissue bud, as visualized by serial sections analysis (Fig. 1A). At e13.5 and e15.5, the vessels had adopted a honeycomb pattern with endothelial cells encircling the base of nascent epithelial branches, as shown by confocal projections; epithelial bulges protruded across the endothelial network forming branch tips that were at some distance from the endothelial cells (Fig. 1A). These morphogenetic changes were faithfully recapitulated in vitro. Indeed, pancreatic explants isolated at e12.5 and further cultured for 1 day on a filter showed the same topological relationship, with tip cells expressing CPA bulging out of the epithelium across the network of endothelial cells (Fig. 1B and Supplementary Fig. 1). Therefore, during pancreas morphogenesis, the endothelial cells initially surround the epithelial mass of the pancreatic bud, then form a honeycomb network embracing epithelial trunk cells, while remaining distant from tip cells.

VEGF-A is expressed in the pancreatic epithelium

Since VEGF-A is a major regulator of blood vessel development, we investigated by in situ hybridization whether the expression pattern of this growth factor and, VEGFR2, the main VEGF-A receptor, correlated with the spatial organization of endothelial cells around the pancreatic epithelium (Fig. 2). *Vegfa* expression was detected in the epithelial cells of the pancreatic bud as early as e11.5, while *Vegfr2* (also called *Flk1*), in an adjacent section, revealed a closely apposed reticular pattern, with no evidence for expression in the pancreatic epithelium itself (Figs. 2A,B and A′B′). At e13.5, *Vegfa* expression became heterogeneous in the pancreatic epithelium, but predominant in trunk cells, and vanishing in tip cells, with *Vegfr2* still restricted to endothelial cells (Figs. 2CD and C′D′). These observations were supported by Q-RT-PCR analysis. E14.5 Pdx-1-GFP mice were used to separate by FACS GFP-positive (pancreatic epithelium) from GFP-negative (non-epithelial) cells. The enrichment of Pdx-1 and of the epithelial marker E-cadherin, and their near absence in the non-epithelial fraction (GFP-), demonstrated the cell sorting efficiency. In the non-epithelial cells, the expression of VEGFR2 was enriched to the same proportion as the endothelial marker VE-cadherin, and the mesenchymal marker FGF-10, indicating lack of epithelial expression of VEGFR2 (Fig. 2E). Similar findings were made for VEGFR1 and VEGFR3. To better visualize the distance between tip cells and endothelial cells, adjacent sections from an e15.5 pancreas were costained with either *Vegfa* or *Vegfr2* and the tip/acinar cell marker CPA (Figs. 2F and F′). *Vegfa* staining was predominantly found in the trunk epithelial cells but not in the CPA-positive tip cells. Endothelial cells, detected by *Vegfr2* labeling, were mainly found adjacent to the trunk epithelial cells, at a distance from the tip cells. Similarly when e12.5 pancreatic explants were cultured for 2 or 3 days on filters, a similar expression profile was observed: epithelial trunk cells were in close proximity of endothelial cells, whereas tip cells were more distant (Figs. 2 G, G′ and G″). We concluded that VEGF-A expression is regionalized during branching morphogenesis and that the predominant expression of VEGF-A in trunk cells correlates well with the predominant location of endothelial cells near the trunk cells, at a distance from the tip cells. The expression pattern of VEGFR2 also suggested that VEGF-A signaling was targeting pancreatic endothelial, not epithelial cells.

Epithelium-derived VEGF-A controls blood vessel and acinar development in vivo

To test for a role of VEGF signaling and blood vessel development on pancreatic differentiation, we performed loss- and gain-of-function experiments in vivo. We first inhibited VEGF-A production by pancreas-specific inactivation of floxed *Vegfa* alleles. To this end, Pdx1-Cre mice, which express Cre recombinase exclusively in the pancreatic epithelium, from e11.5 onwards, were mated with Vegfa-loxP mice. Embryos with homozygous pancreas-specific inactivation of the *Vegfa* allele were analysed at e15.5 (Pdx1-Cre;Vegfa-loxP)(Gerber et al., 1999; Gu et al., 2002). The recombination efficiency was evaluated by in situ hybridization with the *Vegfa* probe (Figs. 3A, B): in Pdx1-Cre;Vegfa-loxP embryos, VEGF-A staining was decreased to background levels throughout the epithelium, indicating that Cre-mediated recombination of the *Vegfa-loxP* alleles was effective. However, some expression of VEGF-A persisted locally, which indicates that Cre-mediated inactivation of the *Vegfa-loxP* alleles was mosaic. The reduction of VEGF-A

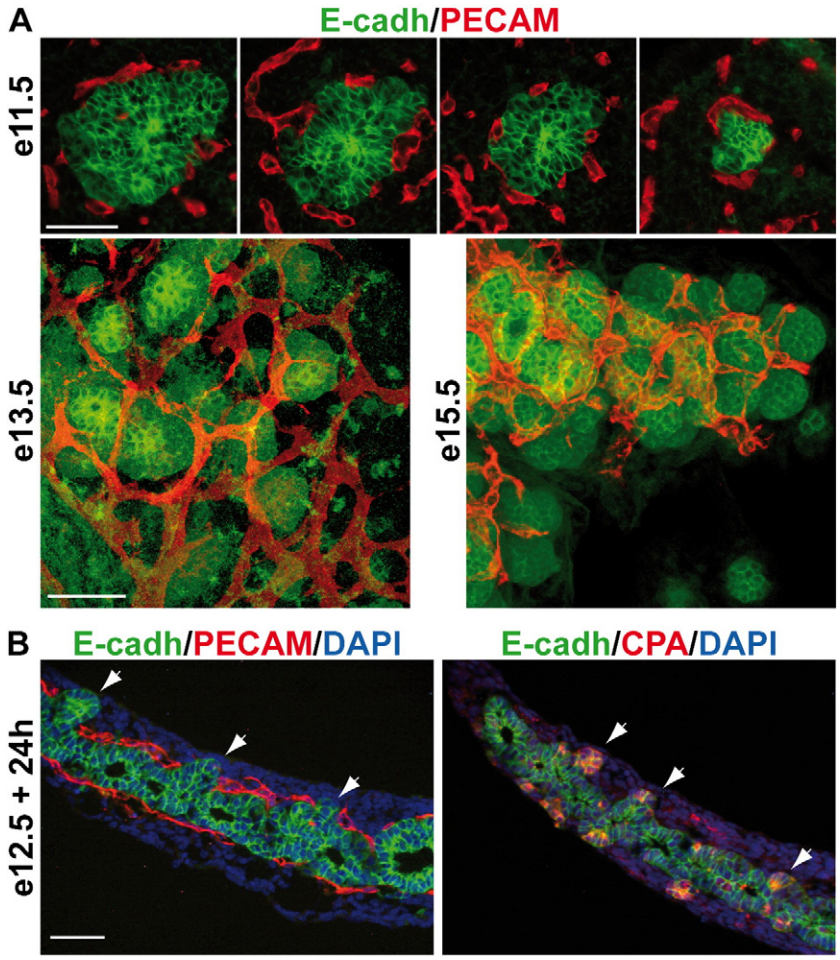

Fig. 1. A dense network of blood vessels surrounds the developing pancreatic epithelium. (A) In vivo: immunolabeling of serial sections of control pancreas from e11.5 embryos. At the initial pancreatic bud stage (e11.5), endothelial cells (PECAM+) surround the epithelial cell mass (E-cadh+). During branching morphogenesis at e13.5 and e15.5, the endothelial cells adopt a honeycomb pattern intertwined with epithelial outgrowths and are predominantly located near trunk cells, at a distance from the tip cells, as shown by confocal projections. (B) Explants: a similar relation between endothelial and epithelial cells is found when e12.5 pancreatic explants are cultured for 24 h. The bulging branch tips identified by CPA (white arrowheads) outgrow endothelial cells in an adjacent section. Scale bars = 50 μm.

expression was associated with hypoplasia of the endothelial network, as revealed by the punctate pattern of PECAM staining in sections (Figs. 3E, E′, F, F′), and three-dimensional reconstruction using multiphoton microscopy (Figs. 3C, D). Moreover, quantification of the endothelial area on sections confirmed the overall reduction in blood vessel density in mutant embryos: the endothelial cell area was 12.1 ± 1.0% of total section area (mean ± SEM, $n = 9$) in wild-type pancreas and 6.9 ± 0.3% in mutants ($n = 14$; $p < 0.001$). However, hypoplasia of the endothelial network was uneven, in line with the mosaic inactivation of *Vegfa-loxP*, decreasing locally to a vessel density down to 1/3 of normal values (3.8 ± 0.4%; $n = 14$; $p < 0.001$) (Fig. 3F′).

Our initial observations of the Pdx1-Cre;Vegfa-loxP mice suggested that acinar development might be affected. Therefore, sections were immunostained to detect the acinar differentiation markers CPA and amylase. The results were quantified by measuring the area of acinar marker expression, normalized for that of the epithelial marker E-cadh. No significant effect on overall acinar differentiation was monitored in Pdx1-Cre;Vegfa-loxP pancreata (CPA/E-cadh ratio = 83.4 ± 3.8; mean ± SEM; $n = 14$) as compared to controls (CPA/E-cadh ratio = 86.7 ± 4.9; $n = 9$; NS), despite the global reduction in blood vessel density. However, in selected regions with lowest vessel density acinar differentiation significantly increased (CPA/E-cadh ratio = 93.6 ± 4.5; $n = 14$; $p < 0.004$). This was also monitored by measuring the Amy/E-cadh ratio, which was 82.2 ± 5.2 in selected areas of the transgenic pancreata as compared to 68.9 ± 4.6 in total pancreas ($n = 14$; $p < 0.008$), pointing to the possibility that endothelial cells repress acinar differentiation (Fig. 3H).

We then generated gain-of-function transgenic embryos overexpressing VEGF-A in the tip cell region (Ptf1a5'enh/Ela1p-Vegfa). Expression of the transgene was analysed by *in situ* hybridization at e15.5 (Figs. 3I, I′). Four embryos expressed high levels of *Vegfa* in most of the tip cells. To visualize blood vessels, the *Vegfr2* probe was hybridized on adjacent sections. This revealed a dramatic change in the organization of the blood vessels in the pancreas of the 4 transgenic embryos. In addition to their normal localization around trunk cells, blood vessels now surrounded the *Vegfa*-expressing acini, demonstrating that endothelial cell localization depends on VEGF-A expression pattern (Figs. 3 I, I′). Pancreatic differentiation was then analysed in these hypervascularized transgenic pancreata. Although acinar structures were formed at the extremities of the pancreatic epithelium, we observed a massive reduction of amylase staining (Figs. 3K, K′). Expression and localization of Ngn3 and of insulin was normal in the transgenic pancreata at e15.5 (not shown). These data indicate that epithelium-derived VEGF-A dictates endothelial cell recruitment and localization, and that in turn blood vessels repress acinar differentiation.

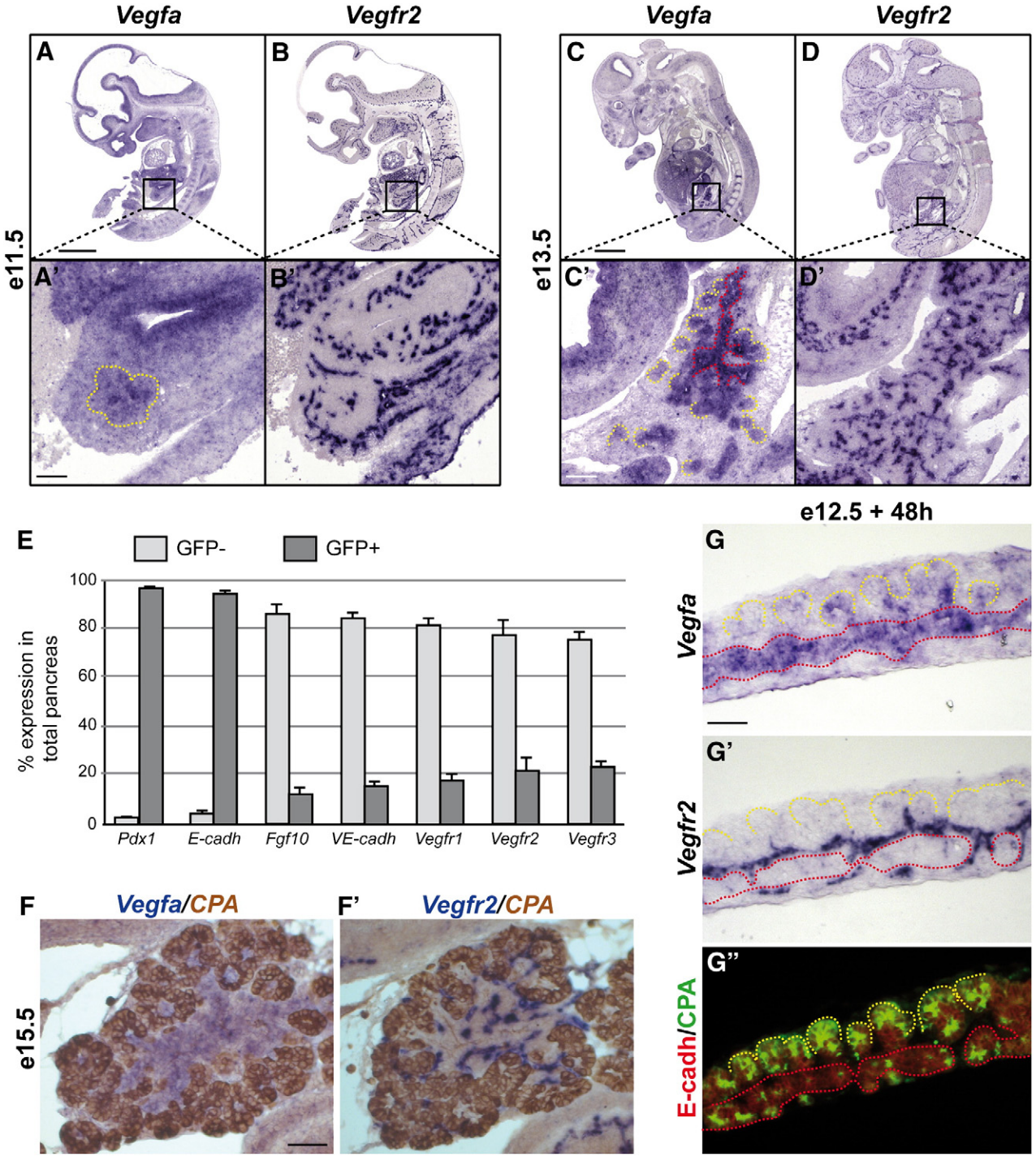

Fig. 2. Epithelial trunk cells express VEGF-A and recruit VEGFR2+ endothelial cells. (A–D) In vivo: In situ hybridization on two serial e11.5 embryo sections show expression of *Vegfa* in the pancreatic bud (yellow dotted line), and *Vegfr2* in endothelial cells around the bud (A, A′, B, B′). At e13.5 (C, C′, D, D′), *Vegfa* shows a higher expression in the trunk cells (red dotted line) as compared to the epithelial outgrowths (yellow dotted line). (E) *Vegfrs* are expressed in the non-epithelial (FACS-sorted GFP−) cell population enriched in VE-cadherin and FGF-10 and depleted in E-cadherin and Pdx1. (F,F') In vivo: In situ hybridization coupled to immunohistochemistry on two serial e15.5 embryo sections show expression of CPA (brown) at a distance of epithelial trunk (*Vegfa*; blue) and endothelial (*Vegfr2*; blue) cells. (G,G′,G″) Explants (3 serial sections): A similar expression pattern of *Vegfa* and *Vegfr2* is found in e12.5 pancreatic explants cultured for 48 h. Trunk cells (red dotted line), highly expressing *Vegfa,* are surrounded by *Vegfr2* + cells. The epithelial outgrowths (yellow dotted line), selectively express CPA. Scale bars in A–D = 1000 μm; in A′–D′ = 100 μm; in F, G, scale bar = 50 μm.

Endothelial cell development in cultured pancreatic explants depends on VEGF-A signaling

To further investigate the role of endothelial cells during pancreas development, we studied pancreatic explants, as these could be treated with recombinant VEGF-A or the VEGFR signaling inhibitor, SU5416. When e12.5 pancreatic explants were cultured for 2 days in the presence of SU5416 endothelial cells disappeared (E-cad/PECAM labeling; Figs. 4A, A′, B, B′). This effect involved apoptosis as suggested by the activation of caspase-3 (Fig. 4G). Likewise, oxindole and PTK787, two unrelated VEGFR2 inhibitors caused disappearance of PECAM expression (Supplementary Fig. 2). Conversely, addition of VEGF-A to the culture medium increased the density of endothelial cells (Fig. 4C′) in a concentration-dependent manner (Supplementary

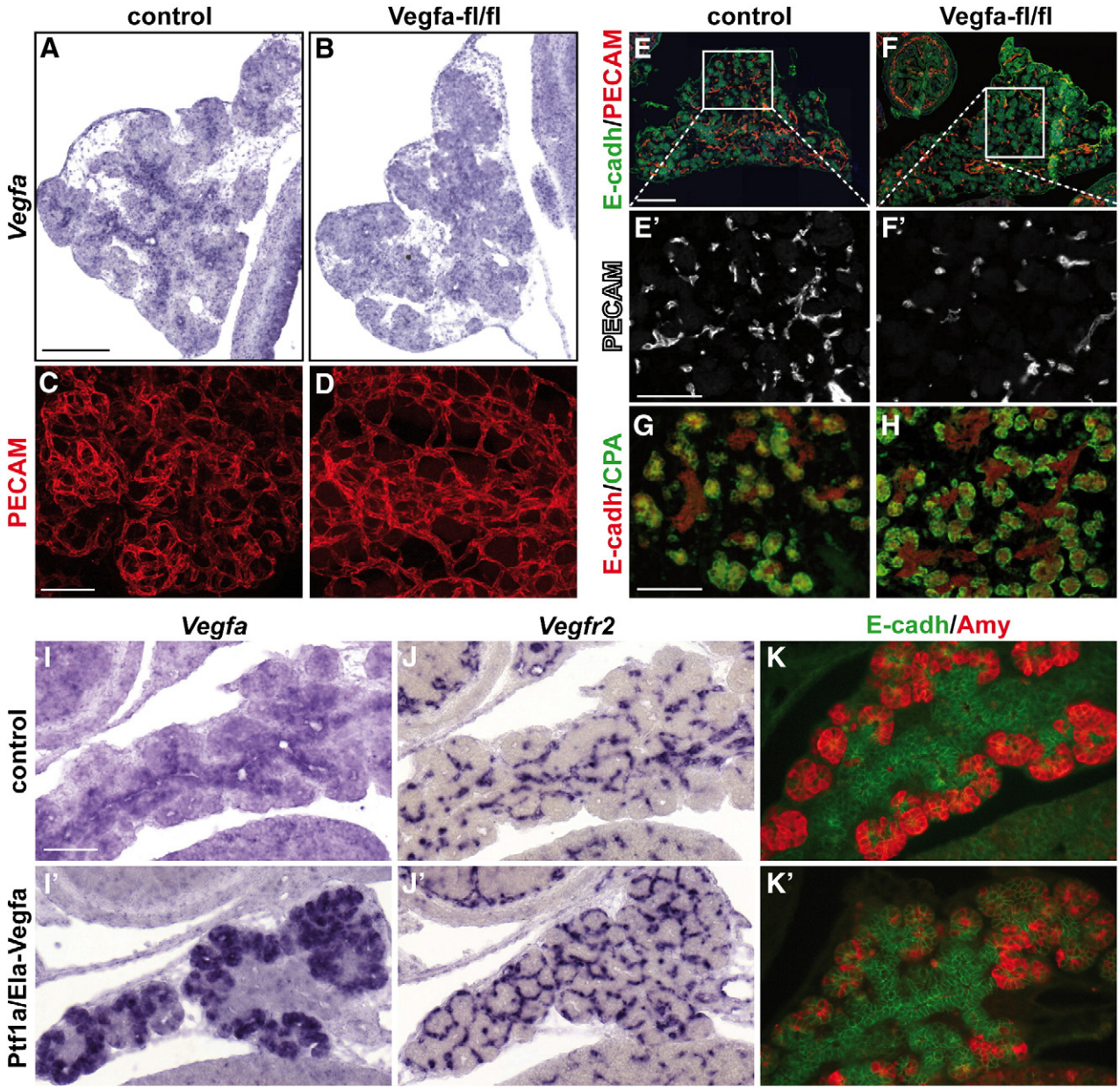

Fig. 3. VEGF-A modulation affects endothelial cell development and acinar differentiation. (A, B) In situ hybridization shows reduced expression of *Vegfa* in e15.5 Pdx1-Cre;Vegfa-loxP (Vegfa-fl/fl) pancreas, as compared to controls. (C–F) Blood vessel density is reduced in Pdx1-Cre;Vegfa-loxP pancreas, as revealed by three-dimensional reconstructions of the endothelial network (C, D) and by punctate immunoreactivity for PECAM on (E, F) selected regions of the pancreas (E, E′, F, F′). (G, H) Increased CPA immunolabeling in selected region of Pdx1-Cre;Vegfa-loxP pancreas. (I, J) In situ hybridization shows increased expression of *Vegfa* in the tip cells of e15.5 Ptf1a-Ela/Vegfa pancreas (I′), and the associated increase in endothelial cell density around the tips (J′), as compared to controls (I, J). (K, K′) Reduced amylase immunolabeling in Ptf1a-Ela/Vegfa transgenic pancreas. Scale bars in A, B, E, F = 200 μm; in C, D, E′, F′, G–K = 100 μm.

Fig. 3). Q-RT-PCR experiments revealed that expression of the endothelial marker VE-cadherin was affected within 12 h of treatment (Fig. 4H). The pancreatic epithelium was also strongly affected by the addition of SU5416 or of VEGF-A, which respectively led to fewer but larger epithelial buds, or less densely packed epithelium (Figs. 4A–C). Confocal analysis of explant sections confirmed the absence of endothelial cells in the SU5416-treated culture and their increase in VEGF-A-treated explants (Figs. 4D–F). Moreover, in explants treated with VEGF-A, the epithelium became almost completely trapped in the endothelial network. Taken together, these results indicated that VEGF signaling regulates the number of endothelial cells and their spatial relationship with the pancreatic epithelium.

Endothelial cells regulate pancreatic cell differentiation

We next examined the expression of acinar differentiation markers in explants in which endothelial development has been modulated. In SU5416-treated explants, i.e. in the absence of endothelial cells, the number of CPA + cells and of Ptf1a + cells was increased after 2 days of treatment (Figs. 5A, B). This increase was restricted to the epithelial outgrowths and was not observed in trunk cells (Fig. 5A). Since CPA and Ptf1a are markers of both pluripotent tip cells and acinar cells, we further assessed the expression of the acinar-specific marker amylase. As shown in Fig. 5A, CPA + cells also expressed amylase and thus corresponded to acinar cells. As predicted, treating explants with VEGF-A, which induced endothelial

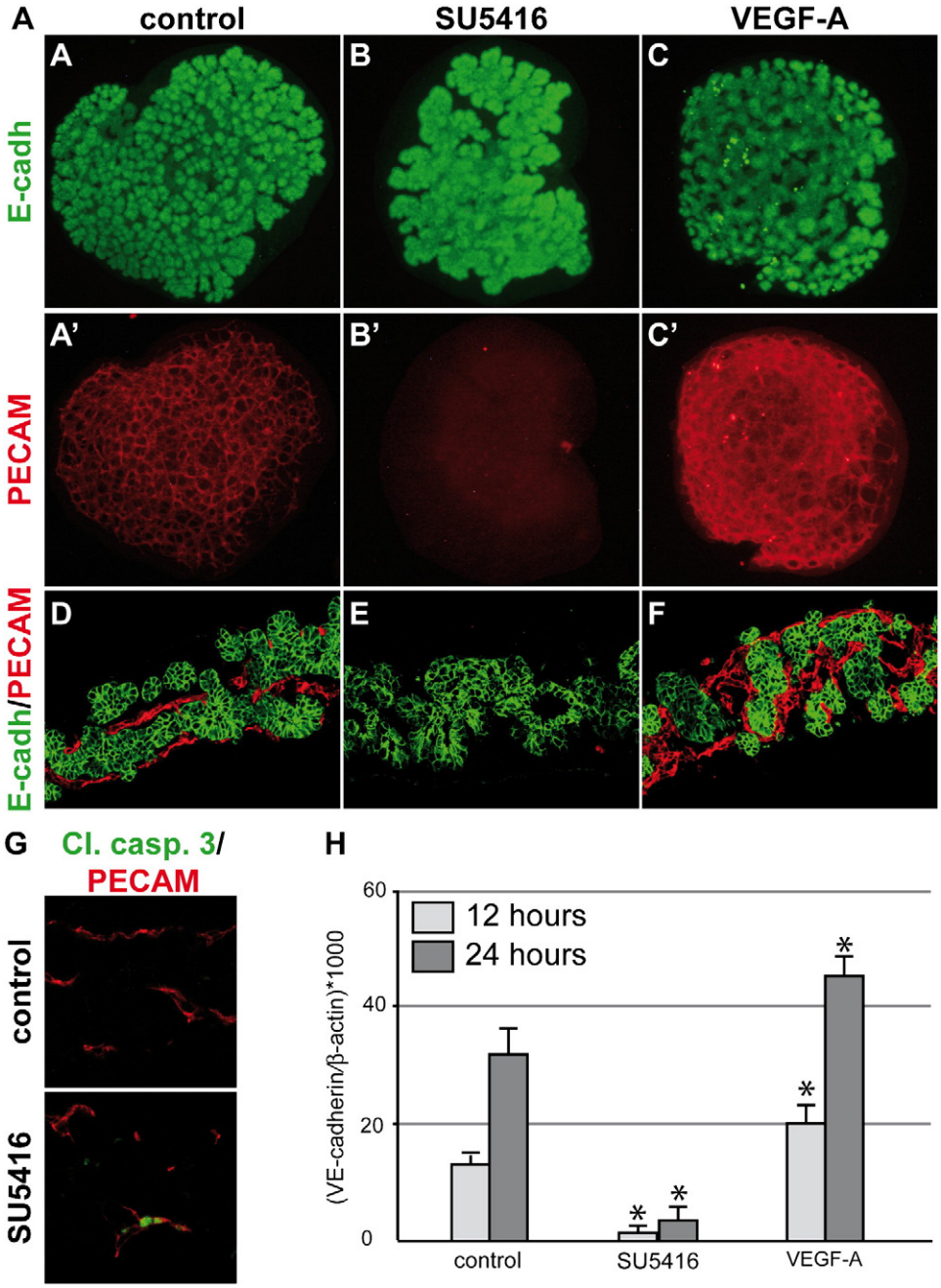

Fig. 4. Modulation of VEGF-A signaling in explants affects endothelial cell density. (A–F) Immunolabeling of whole-mounts (A, A′–C, C′) and sections (D–F) of e12.5 explants cultured for 48 h shows either absence or increased density of blood vessels when VEGF-A signaling is inhibited by SU5416 or stimulated by VEGF-A, respectively. (G) A 4-hour treatment with SU5416 induces endothelial cell apoptosis (cleaved caspase-3; green). (H) Treatment with SU5416 decreases, while addition of VEGF-A stimulates *VE-cadherin* expression in cultured explants, as measured by Q-PCR ($^*p<0.05$ by *t* test).

cell development, had effects opposite to those of SU5416, as shown by decreased expression of CPA and amylase (Fig. 5C). In line with our in vivo observations, these data indicated that a reduction in endothelial cell numbers promotes acinar development, whereas an increase represses acinar differentiation.

To get insight into the mechanism by which endothelial cells repress acinar differentiation, we monitored gene expression in cultured explants by Q-RT-PCR (Figs. 5D, E). Inhibition of VEGF-A signaling by SU5416, which rapidly inhibited endothelial development (see Fig. 4B), induced concomitantly the expression of Ptf1a, RBP-J and RBP-JL, indicating that the acinar transcriptional program was rapidly promoted after repression of VEGF-A signaling (Fig. 5D). Conversely, stimulation of VEGF-A signaling by treatment with VEGF-A induced endothelial development and repressed the expression of these acinar markers after 24 h: the effect of VEGF-A on acinar development was thus slightly delayed as compared with the effect of SU5416 (Fig. 5E). Taken together, these observations indicate that modulation of VEGF-A signaling in endothelial cells impacts on Ptf1a expression in the epithelium, more specifically in the epithelial outgrowths, and not in central epithelial cells (Fig. 5A). The expression of Myc, which is required for exocrine development and is stimulated by Wnt signaling, was not affected by treatment with SU5416 or VEGF-A (Figs. 5D, E). Similarly, Exdpf, which is required for exocrine development in Zebrafish, remained unchanged (Figs. 5D, E). Since Notch signaling is also involved in exocrine differentiation, we measured the expression of the Notch target genes, Hes-1, Hey-1 and

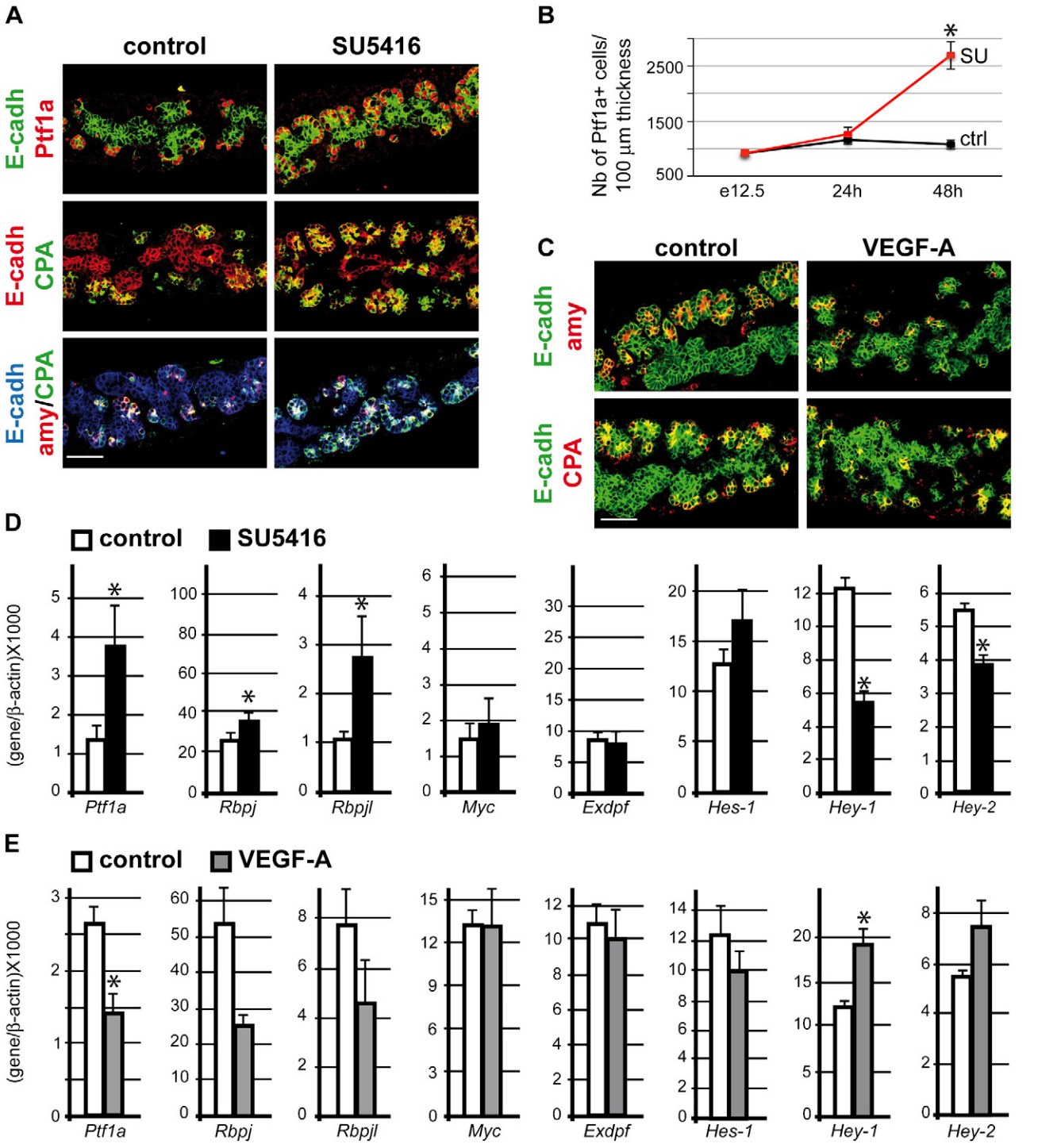

Fig. 5. Endothelial cells repress acinar differentiation in vitro. (A) Increased immunolabeling of Ptf1a, CPA and amylase in SU5416-treated explants. (B) Quantification of Ptf1a + cells in SU5416-treated explants. (C) Decreased immunolabeling of amylase and CPA after 96 h in the presence of VEGF-A. (D, E) Q-PCR profiling in explants cultured for 12 h (D) and 24 h (E). Treatment with SU5416 increases, while VEGF-A decreases the expression of *Ptf1a*, *Rbpj* and *Rbpjl* (*$p<0.05$ by *t* test). Treatment with SU5416 decreases the expression of Hey-1 and Hey-2, while VEGF-A increases that of Hey-1 (*$p<0.05$ by *t* test). The expression of *Hes-1* (measured at 24 h in D and E), *Myc* and *Exdpf* is unaffected. Scale bars in A and C = 50 µm.

Hey-2 upon treatment with SU5416 or VEGF-A. The expression of Hes-1 was not affected, but that of Hey-1 and Hey-2 was reduced by SU5416 and increased by VEGF-A (Figs. 5D, E). Together, these data suggest that blood vessels control exocrine differentiation by modulating Ptf1a expression and Notch signaling.

We also investigated whether modulating VEGF-A signaling affected epithelial cell proliferation or survival. Explants were cultured for 2 days and proliferation of epithelial cells was visualized by co-staining for phospho-histone H3 (pHH3) and E-cadh. No significant effect on proliferation was seen in SU5416-treated explants as compared to controls, whereas treating explants with VEGF-A reduced proliferation (Supplementary Table 3). The effect of VEGF-A was not specific to acinar cells, since the pHH3 + /E-cadh + cells were evenly distributed throughout the epithelium, and not restricted to epithelial outgrowths (data not shown). The lack of cell-type specific proliferation and the inconsistency between the

proliferation rates measured in SU5416- or VEGF-A-treated explants led us to conclude that proliferation is not essential for VEGF-A-controlled acinar development. With regard to cell survival, active caspase-3 staining of sections from control and SU5416-treated explants showed a very low number of apoptotic cells. This number increased upon treatment with SU5416 but remained low (Supplementary Table 3). An increase in apoptosis when VEGF-A signaling is inhibited, cannot explain the concomitant increase in acinar cell numbers.

The mosaic phenotype of Pdx1-Cre;Vegfa-loxP embryos precluded a reliable analysis of endocrine differentiation. Indeed, endocrine cells are much less abundant than acinar cells, and analysis of low numbers of endocrine cells in selected pancreatic regions could produce biased data. Endocrine development was thus analysed on pancreatic explants. The proendocrine marker, Ngn3, was readily immunolabeled in the nuclei of trunk cells in control conditions but had almost vanished after 2 days in the presence of SU5416 (Fig. 6A). The number of cells expressing the late endocrine marker Pax6 was also reduced, further supporting that SU5416 inhibits endocrine differentiation (Fig. 6A). Surprisingly, VEGF-A did not stimulate endocrine differentiation, since expression of Ngn3 and Pax6 remained unchanged (Fig. 6B). The number of Ngn3+ cells in control explants (182.9 ± 67.2) was not statistically different from that in VEGF-A-treated explants (148 ± 8.1). These data suggested that endothelial cells were required but not sufficient to induce endocrine differentiation in cultured explants.

Sox9 marks a progenitor population and is essential for endocrine but not for exocrine development (Seymour et al., 2008; Seymour et al., 2007). At e12.5 in vivo, Sox9 expression is expressed throughout the epithelium, including in tip and trunk cells (Zhou et al., 2007). Later, it becomes restricted to centrally located epithelial cords (Seymour et al., 2007). This was also the case in cultured explants (Fig. 6D). Treatment of explants with SU5416, significantly decreased Sox9 mRNA levels after 24 h (Fig. 6C). By immunofluorescence, Sox9 labeling was also decreased in both epithelial outgrowths and trunk cells (Fig. 6D). Treatment with VEGF-A did not affect significantly the expression of Sox9 (data not shown). These data suggest that VEGF-A signaling is required but not sufficient for Sox9 expression. Considering that the SU5416-mediated repression of Ngn3 and Sox9 occurs simultaneously and is restricted to trunk cells, we suggest that VEGF-A signaling in endothelial cells is required to maintain the Sox9+ progenitor pool and its endocrine derivatives. An effect on Ngn3+ cells, independently from Sox9, cannot be excluded.

We conclude that, during pancreatic development, VEGF-A is secreted by the epithelium and required for recruitment, development and function of pancreatic endothelial cells. In return, endothelial cells signal back to the growing epithelium so as to repress Ptf1a and exocrine differentiation. Endothelial cells are also required to maintain the centrally located endocrine progenitors.

Discussion

In the present work, we first show how endothelial cells develop and localize around the pancreatic epithelium during pancreas development. When the epithelial bud undergoes branching remodeling, the endothelial cells become preferentially positioned close to the trunk cells of the epithelium, while remaining at a distance from the branch tips where exocrine cells differentiate. The preferential location of endothelial cells near trunk cells correlates with the predominant expression of VEGF-A in the latter cells. Inactivation of

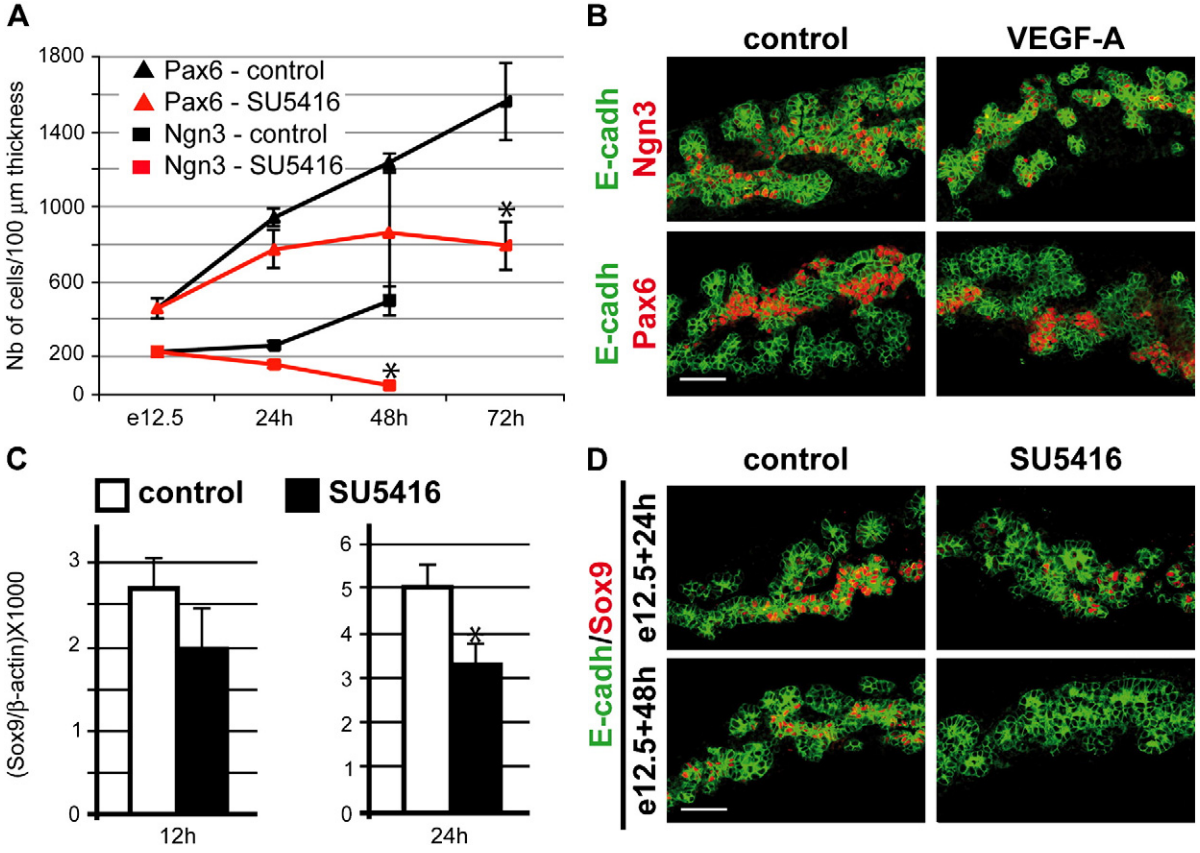

Fig. 6. Endothelial cells are required for endocrine differentiation in vitro. (A) Treatment of explants with SU5416 decreases the number of Ngn3+ cells and slows down the expansion of Pax6 + cells. (B) Addition of VEGF-A does not affect Ngn3 and Pax6 immunolabeling. (C, D) SU5416 decreases expression of Sox9 in explants as shown by Q-PCR (C) and immunolabeling (D) (*$p<0.05$ by t test). Scale bars in B and D = 50 µm.

floxed *Vegfa* alleles in the pancreatic epithelium reduced the number of endothelial cells and increased that of acinar cells. Overexpression of a *Vegfa* transgene in the tip cells induced endothelial cell recruitment around the tips and this decreased acinar differentiation. Similarly, stimulation of VEGF-A signaling in cultured pancreatic explants induced endothelial cell development and repressed acinar development. The opposite effects were seen after inhibition of VEGF-A signaling. Therefore, our data suggest reciprocal interactions whereby endothelial cells organize as a meshwork in response to epithelial-derived VEGF-A, and signal back to limit acinar cell differentiation.

The remodeling of the endothelial network resulting from inactivation of the *Vegfa* alleles, or from overexpression of *Vegfa* specifically in the tip cells, indicates that this signaling factor is a key regulator of the spatial organization of endothelial cells during pancreas development. Extracellular matrix could also participate in the regulation of endothelial regionalization. Indeed, we found that laminin is more abundant near the trunk cells than around the tip cells. However, treating explants with SU5416 inhibited vessel development and reduced laminin production near the trunk cells, thereby abolishing the difference in laminin deposits between the tip and trunk areas (data not shown). Considering that the laminins around β-cells of the islets of Langerhans are produced by the endothelial cells (Nikolova et al., 2006), we suggest that VEGF-A production is the causal event regulating endothelial organization and that extracellular matrix regionalization is a consequence of endothelial cell localization.

Transcription factors or regulators determining regionalized *Vegfa* expression remain to be identified. VEGF-A's predominant location in trunk cells rather than in epithelial outgrowths may result from differential oxygenation of the epithelial cells. Indeed, a key inducer of VEGF-A expression is hypoxia-induced factor-1, and the latter is predominantly expressed in epithelial trunk cells (http://www.genepaint.org/) while progressively decreasing during development (Heinis et al., 2009). Therefore, we speculate that when the epithelium remodels into a ramified structure, the less oxygenated cells express Hypoxia-Induced Factor-1 and VEGF-A, and stimulate endothelial development in their close proximity until a sufficient oxygenation level is reached. Our analysis of FACS-sorted epithelial and non-epithelial cells indicated that the VEGF receptors were expressed in the non-epithelial compartment and were not detectable at significant levels in the epithelium. Consequently, the close proximity between epithelial trunk cells and the endothelial meshwork is optimal for a reciprocal interaction, and the epithelial phenotype induced by modulation of VEGF-A signaling should result from an indirect effect. We therefore propose that the epithelium controls the development and the spatial organization of the endothelial cells through localized (i.e. by trunk cells) secretion of VEGF-A, and that the endothelial cells signal back to the adjacent epithelial cells by means of an as yet unidentified signaling mechanism to control epithelial differentiation.

The role of endothelial cells was investigated in vivo as well as in cultured explants. In these explants, gain- or loss-of-function experiments could be performed by adding VEGF-A or the pharmacological inhibitor SU5416 to the culture medium. Arguably, SU5416 not only inhibits VEGFR2, the main receptor for VEGF-A, but also the platelet-derived growth factor receptor. However, at the concentration used (5 μM), SU5416 preferentially targets VEGFR2 (IC50 = 1 μM) than the platelet-derived growth factor receptor (IC50 = 20 μM). Moreover, oxindole and PTK787, two other VEGFR2 inhibitors, reproduced the effects of SU5416 on endothelial development and on expression of Ptf1a and CPA. In contrast, neither Glivec, a PDGFR inhibitor, nor PDGF itself modulated Ptf1a expression (not shown). We are confident that the acinar phenotype induced by SU5416 results from an inhibition of VEGFR2 since these effects were opposite to those of VEGF-A. Moreover, the results obtained in cultured explants on acinar development are in line with those obtained with genetic approaches in vivo.

Our data indicate that endothelial cells repress acinar differentiation. This is coherent with the preferential location of the endothelial cells at a distance from the acinar cells and suggests that endothelial cells exert their repressive effect by means of a (or several) secreted factor(s). The latter would either fail to reach the branch tips or would diffuse along a gradient generating high, repressive concentrations near the trunk cells, and low, permissive concentrations near the branch tips. According to this interpretation, excessive endothelial development as a result of treatment with VEGF-A would repress acinar development by two, non-mutually exclusive mechanisms. First, the presence of endothelial cells near the branch tips would reduce the diffusion distance of the secreted factor which then reaches the branch tips at high concentrations. Second, an overall increase in secreted factor would result from the increase in the number of endothelial cells that produce this factor. This implies the need for a tight control of the number and location of endothelial cells, as well as of the timing of their development. Indeed, repression of endothelial development in explants treated with SU5416 did not induce acinar differentiation of all the pancreatic cells. Still, we noted that the acinar buds (Ptf1a + or CPA +; see Fig. 5A) in SU5416-treated explants were larger than those in control explants. Since there is no increase in proliferation, this suggests that trunk cells close to the tip cells had been diverted from their ductal/endocrine fate towards the acinar fate. The rest of the epithelial trunk cells would already be committed to ductal or endocrine differentiation and could not revert their phenotype to the acinar lineage.

The identity of the downstream effectors of endothelial signaling remains unknown. Three candidates do not appear to qualify (Wnt, Exdpf, epithelial–mesenchymal contact), but Ptf1a and Notch signaling are likely candidates. First, our data do not favor the involvement of Wnt signaling in the endothelium-mediated control of exocrine development since the expression of the Wnt target gene Myc was unaffected in our experiments. Second, Exdpf is a signaling mediator identified as a key regulator of exocrine development in the zebrafish; its expression was not affected in our experiments with mouse explants. Third, whereas increased contact between epithelium and mesenchyme promotes duct and acinar differentiation (Crisera et al., 2000; Duvillie et al., 2006; Li et al., 2004; Miralles et al., 1998), VEGF-A reduced the packing of the epithelium, thereby facilitating interactions between the epithelium and mesenchyme (Fig. 4). However, this is associated with repression of acinar differentiation, eliminating the possibility that VEGF-A controls acinar differentiation by regulating interactions between the epithelium and mesenchyme. Conversely, stimulation of VEGF-A signaling and endothelial development repressed Ptf1a expression. In contrast, expression of Ptf1a was upregulated when VEGF-A signaling and endothelial development were inhibited. Since there was no evidence for a specific increase in exocrine cell proliferation that might explain the increase in Ptf1a mRNA levels and in the number of Ptf1a + cells, our data suggest that Ptf1a expression in progenitor cells is the target of endothelial signaling. Our data further suggest an additional mechanism that regulates Ptf1a function. Indeed, treating explants with VEGF-A or SU5416 showed that endothelial development is associated with induction of Hey1 and Hey2. These are Notch signaling effectors known to repress Ptf1a activity (Esni et al., 2004). Therefore, endothelial signaling might repress acinar differentiation by repressing expression and activity of Ptf1a. Our data do not rule out the possibility that endothelial cells signal to mesenchymal cells which would serve as a relay to mediate effects on the epithelium. In collaboration with K. Zaret's team, our group has proposed the existence of such a relay pathway at the onset of pancreas morphogenesis. At this stage, endothelial cells promote survival of mesenchymal cells, which secrete factors that control pancreas morphogenesis and maintain pancreatic differentiation (Jacquemin

et al., 2006). Interestingly, at the stage of pancreas specification, aortic endothelial cells induce Ptf1a expression in the adjacent endoderm (Yoshitomi and Zaret, 2004), suggesting that aortic cells and pancreatic endothelial cells have opposite functions on Ptf1a expression.

Our in vitro experiments also suggested a role for VEGF-A signaling in endocrine development. Previous work has shown that endothelial cells can initiate endocrine differentiation at an early stage of pancreas development (Lammert et al., 2001). Addition of VEGF-A to e12.5 explants or overexpression of VEGF-A in tip cells in vivo did not stimulate endocrine development at e15.5. However, inhibiting VEGF-A signaling in vitro with SU5416 reduced endocrine differentiation, suggesting that VEGF-A signaling and endothelial cells are required but not sufficient for endocrine development. Unfortunately, the mosaic phenotype of Pdx1-Cre/Vegfa-loxP embryos did not allow to reliably evaluate their endocrine phenotype. Since the endocrine compartment represents but a minor fraction of the total pancreas (5%), any selection of affected regions, as performed for the analysis of the exocrine compartment, could generate biased data.

The lack of endocrine stimulation in response to additional VEGF-A and endothelial cell signaling could be explained if all progenitor cells were maximally stimulated by the abundant endothelial cells already present in control conditions. In addition, the number of Sox9-expressing progenitors was decreased in the presence of SU5416, suggesting that endothelial cells may control this progenitor cell type. The reduction in Sox9+ cells may lead to the observed decrease in Ngn3+ progenitors since lineage tracings have demonstrated that Sox9+ cells give rise to Ngn3-expressing cells (Seymour et al., 2007). A word of caution is needed with regard to the use of pharmacological inhibitors, since we cannot formally eliminate the possibility that they can induce non-specific effects on endocrine development. Using VEGFR2 blocking antibodies or soluble VEGFR2 in explant cultures did not allow to obtain strong reduction of endothelial development, thereby preventing further analysis of pancreas differentiation. Despite this limitation, the observation that endothelial cells may send signals required for maintenance and expansion of Sox9+ and Ngn3+ cells seems of importance for in vitro programmed differentiation towards cell therapy of diabetes.

In conclusion, cross-talk between the pancreatic epithelium and the endothelial cells controls the number and spatial organization of the endothelial network closely intertwined around the epithelial branches. This interplay is necessary for appropriate differentiation of acinar cells. In vitro observations further suggest that endothelial cells may also be required to maintain endocrine progenitors.

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2010.08.024.

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