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Osteopontin is a Novel Marker of Pancreatic Ductal Tissues and of Undifferentiated Pancreatic Precursors in Mice

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Abstract

Matricellular proteins mediate both tissue morphogenesis and tissue homeostasis in important ways because they modulate cell-matrix and cell-cell interactions. In this study, we found that the matricellular protein osteopontin (Opn) is a novel marker of undifferentiated pancreatic precursors and pancreatic ductal tissues in mice. Our analysis also underscored a specific, dynamic profile of Opn expression in embryonic pancreatic tissues that suggests the participation of this protein's function in processes involving cell migration, cell-cell interactions, or both. Surprisingly, our analysis of Opn-deficient pancreata did not reveal obvious alterations in the morphology or differentiation of these tissues. Therefore, in embryonic pancreatic tissues, it is possible that other proteins act redundantly to Opn or that this protein's function is dispensable for pancreas development. Finally, the maintenance of Opn expression in pancreatic tissues of adults argues for a possible function of this protein in injury and pathologic responses.

Keywords

Pancreas; osteopontin; mouse; matricellular proteins; development; pancreatic ducts; pancreatic precursor; embryo

INTRODUCTION

The mature mammalian pancreas is a mixed gland composed of endocrine and exocrine tissues that produce and secrete hormones and enzymes required for nutritional balance. In the mature pancreas, the pancreatic exocrine cells produce the precursors of various digestive enzymes (zymogens), which are collected by a system of intralobular and large ducts and then transported to the duodenum, where they are processed to their active form (Slack, 1995). Pancreatic endocrine cells associate into distinctive spherical structures known as the islets of Langerhans. In these islets, the α cells produce glucagon, the β cells produce insulin, the δ cells produce somatostatin, and the PP cells produce pancreatic polypeptide (Slack, 1995). Because the pancreatic hormones maintain blood glucose homeostasis, their function is crucial to metabolism.

Pancreas organogenesis manifests early in vertebrate development as two protrusions (dorsal and ventral) at the posterior foregut region of the embryo (in mice, this occurs between embryonic [E] days 9.5 and 10.5). After formation of these two primordia, the pancreatic epithelium grows intensely, it undergoes branching morphogenesis and in a non-synchronous manner it produces the precursors of endocrine, exocrine, and ductal cells

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(Murtaugh and Melton, 2003; Pictet and Rutter, 1972; Slack, 1995). As demonstrated by the results of numerous in vivo and in vitro studies, the pancreatic epithelium's growth, morphogenesis, and cellular differentiation depend on its interaction with surrounding tissue (Edlund, 2002; Kim and Hebrok, 2001; Murtaugh and Melton, 2003; Wilson et al., 2003). Similarly, evidence gathered from genetic studies supports the notion that some crucial regulators of normal pancreas organogenesis are also key molecular components of pancreatic diseases (Edlund, 2002; Habener and Stoffers, 1998; Kim and Hebrok, 2001; Miyamoto et al., 2003). Therefore identification of the molecular and cellular mechanisms regulating mammalian pancreas development appears necessary, not only to an understanding of the origins of pancreatic dysfunction, but also to the development of tools to improve treatment and prevention of pancreatic diseases (e.g., diabetes mellitus, pancreatitis, or pancreatic cancer).

Cell-cell and cell-matrix interactions play a crucial role in tissue morphogenesis and in homeostasis of adult tissues. The extracellular matrix (ECM) proteins regulate cell function and morphogenesis in important ways because they contribute directly to the organization or physical properties of structures such as fibrils or basal laminae and because they participate in diverse cellular processes. Indeed, the capacity of cells to adhere to the ECM, crucial to cytoskeletal organization and cellular morphology, is also implicated in a cell's ability to proliferate, migrate, survive, or differentiate (Murphy-Ullrich, 2001). The matricellular proteins integrate a separate subclass of matrix proteins that have no structural role, but they contribute to the regulation of cellular homeostasis. Matricellular proteins bind to many cell-surface receptors, the ECM, growth factors, cytokines, and proteases. They also modulate cell-matrix interactions. These structurally diverse proteins include thrombospondins (TSPs) 1 and 2, the tenascins, SPARC (secreted protein, acidic and rich in cysteine or osteonectin), and osteopontin (Opn) (Bornstein and Sage, 2002). In contrast to ECM proteins that generally foster strong cell adhesion, matricellular proteins induce a state of intermediate adhesion that promotes cell motility and that may support cell survival and cell differentiation (Murphy-Ullrich, 2001). The state of intermediate adhesion is characterized by disruption of focal adhesions and reorganization of actin stress fibers. In general, the ability of matricellular proteins to modulate cell adhesion and cytoskeletal organization suggests that they play an important role in developmental and homeostatic processes.

A phosphorylated acidic glycoprotein, Opn can engage a number of receptors, including members of the integrin receptor family (e.g., $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, $\alpha_8\beta_1$ and $\alpha_9\beta_1$) and variants of CD44, the hyaluronic acid receptor. Some Opn interactions involve an RGD (Arg-Gly-Asp) sequence that is also found in various ECM proteins and that binds directly to many integrins (Denhardt et al., 2001). In apparent contradiction to the de-adhesive properties of most matricellular proteins, Opn promotes adhesion of diverse cells to the ECM; however, Opn's interaction with its receptors induces an intermediate state of adhesion that thus enables migration, stimulates specific cellular processes, or does both (Bornstein and Sage, 2002). Although osteopontin has been implicated in a broad range of homeostatic (bone remodeling, tissue debridement) and pathologic (cellular immunity, wound healing, cancer metastasis) events, the precise molecular mechanisms underlying its function remain largely unknown (Denhardt et al., 2001; Gravallese, 2003; Liaw et al., 1998; Mazzali et al., 2002; Rittling and Chambers, 2004).

Gu et al. (2004) previously reported that in early mouse embryos (E10.5) *Opn* transcripts were expressed in the pancreatic epithelium. The expression of Opn in developing pancreatic tissues of mice suggests a possible involvement of this protein's function in some aspects of pancreas organogenesis. Therefore, in this study we sought to characterize in detail the expression of *Opn* mRNA and Opn proteins throughout mouse pancreas ontogeny

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and to determine whether the function of Opn is necessary to pancreas organogenesis by analyzing the pancreatic tissues of Opn-deficient mice.

RESULTS

Dynamic Expression of Osteopontin During Mouse Pancreas Ontogeny

To determine the temporal and spatial pattern of Opn expression during mouse pancreas ontogeny, we used in situ hybridization and immunohistochemical techniques. Our in situ analyses revealed low levels of *Opn* transcripts in pancreatic tissues of embryos as early as E10.5 (data not shown). Two days later, the expression of *Opn* mRNA increased substantially, and these transcripts were broadly distributed throughout most of the pancreatic epithelium, with one exception: some large cell clusters located at the periphery of the epithelial tissue were devoid of *Opn* mRNA (Fig. 1A). At E14.5, most pancreatic cells near the epithelial lumen expressed *Opn* mRNA; in contrast, no *Opn* transcripts were detected at the tip of the branches or in cell aggregates on the basolateral side of this tissue (Fig. 1B). After E16.5, *Opn* mRNA's expression became restricted to cells in the ductal portion of the embryonic pancreas (Figs. 1C, D). A similar temporal pattern of expression was also observed for Opn proteins. For example, in the pancreata of embryos isolated at E10.5, we observed Opn immunoreactivity in the cytoplasm of a few cells or in the apical side of cells located towards the lumen of the epithelium (Fig. 1E). From E12.5 until approximately E14.5, the number of pancreatic epithelial cells expressing Opn increased considerably and in the majority of these cells Opn proteins appeared distributed throughout the cytoplasm (Fig. 1F). In contrast, towards the end of gestation (E18.5) all of the pancreatic Opn immunoreactivity appeared deposited on the apical side of ductal epithelial cells (Fig. 1G). In the pancreata of newborn mice we found that the expression of Opn became restricted to ducts and centroacinar cells, and we did not detect these proteins in the endocrine or exocrine compartments (Fig. 1H and data not shown).

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In the pancreas of adult mice, Opn proteins remained expressed in the ductal tissue and they were also detected in the endocrine islets of Langerhans (Fig. 2A). In this organ, low levels of Opn immunoreactivity appeared deposited on the apical side of both intralobular ducts (Fig. 2B) and large ducts (Fig. 2C). In contrast, in pancreatic islet cells Opn proteins were diffusely distributed in the cytoplasm (Fig. 2A, D–F). In most islet cells, Opn proteins co-localized with the β cell markers Pdx1 (Fig. 2D) or insulin (Fig. 2E), although it also co-expressed with the δ cell marker somatostatin in a small number of cells (Fig. 2F).

Numerous Pancreatic Cells Express Opn in the Cytoplasm Between E12.5 and E14.5

A detailed analysis of Opn protein distribution in embryonic pancreatic tissue of mice revealed two distinct patterns of expression. During most of pancreas organogenesis (i.e., between E10.5 and post-natal day 2), we noticed deposition of Opn proteins on the apical side of cells located towards the lumen of the epithelium (Figs. 1E–H and 3). However, in the pancreas of embryos dissected between E12.5 and E14.5 we also found that most Opn-immunopositive cells expressed this protein broadly in the cytoplasm (Opn_{cytopl}^{+} cells). Many of these Opn_{cytopl}^{+} cells appeared to be stretching between the luminal and basolateral sides of the epithelium (arrows in Figs. 3A, B, D and 4B, E), often contacting the laminin-rich basal membrane (Fig. 3C), whereas other Opn_{cytopl}^{+} cells seemed to locate only to the basolateral side of this tissue (arrowhead in Fig. 3A).

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The Opn_{cytopl}^{+} cell population of the embryonic pancreas is remarkable in another way: most of these cells seemed to represent undifferentiated epithelial precursors. In fact, in the entire pancreas of E13.5 embryos we found that only a few cells (perhaps no more than 4 or 5 epithelial cells) co-expressed Opn_{cytopl} and Ngn3 (a marker of endocrine specification;

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Fig. 4B), Opn_{cytopl} and p48 (a marker of exocrine specification; Fig. 4D) or Opn_{cytopl} and elastase (an exocrine marker; Fig. 4E), and we never observed co-localization of Opn_{cytopl} with various markers of endocrine differentiation (e.g., Pax6, Islet 1 or glucagon; Fig. 4A,C; data not shown) in the pancreata of embryos isolated between E12.5 and E14.5. In contrast, some Opn_{cytopl}^{+} cells seemed to represent proliferating cells because they incorporated bromodeoxyuridine after a 1-hour pulse (Fig. 3D).

In summary, our analysis of Opn expression throughout mouse pancreas ontogeny revealed a dynamic pattern of this protein's cellular distribution that also suggests a distinctive requirement of Opn function in specific pancreatic cell types of embryos and adults.

Expression of Opn in Other Organs of the Developing Gastrointestinal Tract

Osteopontin exists as an immobilized ECM molecule in mineralized tissues and as a cytokine in body fluids (Denhardt et al., 2001). For instance, in adult humans OPN proteins can be deposited as a prominent layer at the cell-luminal surface of various epithelia, including those of the gastrointestinal tract, gall bladder, intrahepatic bile ducts and pancreatic ducts (Brown et al., 1992). In mouse embryos and newborn mice we found that in addition to the pancreas Opn is also expressed in the glandular portion of the stomach and in specific regions of the liver, but not in the duodenum (Fig. 5). In the glandular stomach of embryos isolated at E17.5, we observed a prominent expression of *Opn* transcripts in groups of cells at the base of the developing pits (Fig. 5A). In the glandular stomach of newborn mice, we also detected Opn immunoreactivity in mucin-producing cells in the neck/base of the developing gastric units (Fig. 5B) and in groups of cells probably located in the proliferative compartment of the isthmus (Fig. 5C) (Falk et al., 1994; Kim et al., 2005).

In the liver of E17.5 embryos or newborns, we detected Opn expression in the developing intrahepatic bile ducts, but not in the parenchyma that contains the hepatocytes (Fig. 5D). In the liver of adults the expression of Opn was similar, although the levels of Opn immunoreactivity were substantially lower than those in embryos (data not shown). On the other hand, in the duodenal region of embryos dissected at E18.5, we noticed Opn expression in the terminal region of the pancreatic duct but not in the epithelium of the duodenum (Fig. 5E, F). Overall, the presence of Opn proteins in the epithelium of various digestive organs suggests that this protein's function may be required in the development of these structures, their homeostasis in adult stages, or in both.

Normal Pancreas Development of Opn-deficient Mice

Mice deficient in Opn develop normally, are fertile, and reach adulthood although there is some evidence of alterations in vascular function, wound healing, and bone resorption in such mice due to the lack of Opn function (Liaw et al., 1998; Myers et al., 2003; Yoshitake et al., 1999). However, to our knowledge, no defects have been reported in the development or the physiology of Opn-deficient pancreatic tissues. Therefore, to determine whether the function of Opn is necessary for pancreas organogenesis, we compared the expression of various morphological or cytodifferentiation markers in pancreatic tissues of wild-type newborn mice to that of Opn-deficient newborn mice.

Our results did not show any obvious morphologic defects in the pancreata dissected from wild-type and Opn-deficient newborns. The architecture of the pancreatic epithelium of wild-type and *Opn*-nullizygous newborn mice were similar. In addition, the lack of Opn function did not seem to affect the generation or spatial organization of exocrine, endocrine, and ductal pancreatic tissues (Fig. 6; data not shown). These results suggest that Opn function is dispensable to pancreas organogenesis or that, in these tissues, other proteins compensate for the lack of Opn activity.

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Some candidates that could act redundantly to Opn during organogenesis include other members of the SIBLING (Small Integrin Binding LIgand N-linked Glycoprotein) family of proteins (e.g., bone sialoprotein [Bsp], dentin matrix protein [Dmp] or dentin sialophosphoprotein [Dspp]) (Fisher et al., 2001; Wilson et al., 2005). In developing mice, Opn, Dmp and Bsp appear to be similarly expressed in specific developing organs (e.g., the testes cords). Thus, it is possible that during the formation of these structures the three proteins accomplish analogous functions (Wilson et al., 2005). Another ECM protein (vitronectin [Vn]) could also act redundantly to Opn in developing pancreatic tissues; these two proteins bind to the same integrin receptors, and they are similarly expressed in various embryonic tissues of mice (Liaw et al., 1998). To verify whether Dmp, Bsp, or Vn are also expressed in the developing pancreatic tissue of mice, we performed reverse-transcriptase polymerase chain reaction analyses. For these experiments, we used RNA of pancreatic tissue dissected at E14.5 because we previously determined that the number of Opn$^+$ cells in the pancreas is very abundant at this approximate developmental stage (Figs. 1–3). By using this approach, we identified transcription of *Opn* and *Vn* mRNAs, but not of *Dmp* or *Bmp* mRNAs, in pancreatic tissues of embryos dissected at E14.5 (Fig. 7A).

Our results showing the presence of *Vn* transcripts in mouse embryonic pancreata are important in light of a study conducted by Cirulli et al. (2000) in which the authors demonstrated expression of VN in pancreatic epithelial cells of human fetuses. Therefore, by using immunohistochemical analysis, we sought to compare the expression of Opn with that of Vn in pancreatic tissues of E12.5–E14.5 mouse embryos and to determine whether Vn proteins are still expressed in Opn-deficient pancreata. Our results showed that Vn proteins are expressed in the cytoplasm of many pancreatic epithelial cells of E13.5 wild-type mouse embryos (Fig. 7 B,E). In addition, in these embryos we also detected low levels of Vn expression in cells of the mesenchyme surrounding the pancreatic epithelium (arrowheads in Fig. 7B). More important, our results also uncovered that in E13.5 wild-type embryos Vn and Opn proteins co-localize in the cell membrane or in the cytoplasm of pancreatic epithelial cells (Fig. 7D–F). Furthermore, it is also possible that Opn and Vn could act redundantly during pancreas organogenesis to control similar molecular processes, because the lack of Opn did not affect the expression or the distribution of Vn proteins in Opn-deficient embryonic pancreatic tissues (Fig. 7C).

DISCUSSION

Our results showed a distinctive spatial and temporal pattern of Opn expression during mouse pancreas ontogeny. Specifically, in the pancreata of embryos isolated between E12.5 and E14.5, Opn was expressed in numerous undifferentiated cells scattered throughout most of this tissue. Conversely, late in gestation the expression of Opn became restricted to cells of the developing ducts and to centroacinar cells. In the pancreas of adults, Opn was expressed in some islet cells and in the epithelium of small and large ducts. The cellular distribution of Opn proteins also varied during pancreas organogenesis. For instance, in the pancreas of embryos isolated between E12.5 and E14.5 Opn proteins appeared broadly dispersed in the cytoplasm of epithelial cells. Similarly, in islet cells of the adult pancreas Opn proteins also localized to the cytoplasm. Conversely, in the pancreatic ducts of newborns and adults the expression of Opn proteins was polarized and it was restricted to the apical side of the epithelium.

The specific distribution of Opn proteins in the cytoplasm of pancreatic epithelial cells is intriguing, since some studies have shown that the interaction of intracellular Opn proteins with specific membrane receptors enables cell migration (e.g., the association of Opn with the CD44 receptor of fibroblasts, macrophages and osteoclasts facilitates cell motility) (Denhardt et al., 2001; Fedarko et al., 2004; Suzuki et al., 2002; Zohar et al., 2000; Zhu et

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al., 2004). Hence, Opn could favor the motility of pancreatic epithelial cells via its interaction with specific intracellular receptors. Opn proteins could also modulate the adhesion/migration properties of pancreatic epithelial cells via interacting with integrin receptors. Indeed, integrins are important regulators of pancreas organogenesis since inhibition of integrin's function alters the migration of endocrine progenitors from the ductal epithelium and it also affects islet morphogenesis (Cirulli et al., 2000). Despite these evidences, in the pancreata of Opn-deficient mice we did not observe any obvious defects in the architecture or cellular composition of these tissues. Therefore, it is possible that other proteins (e.g., Vn) act redundantly with Opn in developing pancreatic tissues to control certain aspects of this organ's formation or else, that Opn does not have a major role in the development of pancreatic tissues.

Different studies have shown that most mice lacking the function of a specific matricellular protein have either a grossly normal or a subtle developmental phenotype. However, in many cases these defects became significantly exacerbated when the mutant mice sustained an injury (Bornstein and Sage, 2002). In fact, the study of *Opn*-null mice has revealed important roles for Opn in specific tissues at postnatal stages and in a broad range of homeostatic processes linked by several common themes (e.g., enhanced expression of Opn in response to tissue injury and stimulation of cell motility and cell survival pathways) (Denhardt, et al., 2001; Gravallese, 2003; Liaw et al., 1998; Mazzali et al., 2002). In addition, in certain human malignancies (including pancreatic adenocarcinoma and gastric carcinoma) OPN proteins were found over-expressed and in some cases the elevated expression of this protein correlated with advanced tumor stages or increased invasiveness of the cancer cells (Coppola et al., 2004; Kolb et al., 2005). OPN was also identified previously as a novel autoantigen of patients with insulin-dependent diabetes mellitus (IDDM) and in this study we showed expression of Opn proteins in pancreatic islet cells of wild-type adult mice (Fierabracci et al., 2000). Collectively, these results support the proposal that in the pancreas of adults the function of Opn is likely necessary for this organ's repair under pathologic conditions that compromise the integrity or the homeostasis of this tissue (e.g., diabetes, pancreatic cancer or chronic pancreatitis).

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In conclusion, our results showed a specific, dynamic profile of Opn expression in pancreatic tissues that suggests the participation of this protein's function in processes involving cell migration, cell-cell interactions or both at embryonic stages, and in injury and pathologic responses in the adult.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were utilized to analyze the expression of Opn. For timed pregnancies, the day of the plug was counted as 0.5 days. The *Opn* nullizygous mutant mice were originally obtained from Jackson Laboratory (Bay Harbor, ME). The genotyping of *Opn* mutant mice using tail DNA was performed by the polymerase chain reaction (PCR) using *Neo*-specific forward primer, 5′-GAAGCGGGAAGGGACTGGCTGCTA-3′ and reverse primer, 5′-CGGGAGCGGCGATACCGTAAAGC-3′, and *Opn*-specific forward primer, 5′-TTCCAAAGAGAGCCAGGAGA-3′ and reverse primer, 5′-TTGGTTACAACGGTGTTTGC-3′. All experimental protocols were approved by the animal care and use committee at St. Jude Children's Research Hospital.

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Processing of embryos and pancreatic tissues

Tissues of dissected embryos or newborn mice were prepared for immunohistochemical analysis or in situ hybridization by fixation overnight in 4% paraformaldehyde at 4°C.

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Tissues were then immersed in 30% sucrose in phosphate-buffered saline (PBS) overnight at 4°C for cryoprotection, embedded in tissue-freezing medium (Tissue-Tek, Triangle Biomedical Sciences), and cut by a cryostat into sections (8–10 μm for immunohistochemical study and 12 μm for in situ hybridization). Adult mice were perfused manually with cold 4% paraformaldehyde prior to dissecting their digestive tissues, which were then fixed for two more days in 4% paraformaldehyde at 4°C.

Immunohistochemical analysis

Frozen sections underwent immunohistochemical assays. Primary antibodies were the following: rabbit anti–α-amylase (diluted 1:1,000; Sigma); mouse anti-bromodeoxyuridine (IgG isotype; final concentration, 7 mg/ml; Becton Dickinson); rabbit anti-elastase (1:2,000; AbCam); rabbit anti-glucagon (1:50, Zymed); guinea pig anti-insulin (1:250; DAKO); rabbit anti-mucin (1:50; Santa Cruz Biotechnology); rabbit anti-laminin (1:2,000; Sigma); guinea pig anti-Ngn3 (1:2,000; provided by M. German); rabbit anti-Nkx6.1 (1:1,000; provided by P. Serup); goat anti-osteopontin (1:25; R&D Systems); rabbit anti-p48 (1:400; provided by H. Edlund); rabbit anti-Pax6 (1:1,000; Covance Research Products); rabbit anti-Pdx1 (1:1,000; provided by C. Wright); rabbit anti-vitronectin (1:100; Chemicon); rabbit anti-somatostatin (1:100; Zymed); and rat anti–uvomorulin/E-cadherin (1:5,000; Sigma). The following secondary antibodies (diluted 1:200) were used for detection: Cy3-conjugated donkey (anti–goat, anti–guinea pig, anti–mouse, anti–rabbit or anti–rat) IgG from Jackson ImmunoResearch Laboratories, Inc.; Cy5-conjugated donkey anti–rat IgG (Jackson ImmunoResearch Laboratories, Inc); Alexa 488–conjugated goat anti–rabbit IgG (Molecular Probes); Alexa 488–conjugated donkey anti–goat IgG (Molecular Probes). Biotinylated horse anti–goat IgG (Vector Laboratories) was detected by using the VECTASTAIN Elite ABC kit (Vector Laboratories). For nuclear staining, sections were covered with mounting media containing 4′, 6-diamidino-2-phenylindole (DAPI) or TO-PRO-3 iodide (carbocyanine monomer), or were counterstained with propidium iodide (Sigma). Images were obtained either with the Zeiss Axioskop 2 microscope or with a Leica TCS confocal laser–scanning microscope. Adobe Photoshop version 7.0 (Adobe Systems, Inc.) was used to process the images.

In situ hybridization

The digoxigenin-labeled *Opn* sense or antisense mRNA probes were transcribed in vitro by using a plasmid containing 300 bp fragment of *Opn* cDNA inserted into the PGEMT-easy vector (Promega). The probes were used for non-radioactive in situ hybridization on 12-μm frozen sections as previously described (Wang et al., 2004).

BrdU staining

Pregnant females were injected with BrdU (100mg/g of body weight) at E11.5 of gestation. Embryos were dissected 1 hour later and processed for cryosectioning as previously described. Frozen sections were incubated in blocking solution (20% fetal bovine serum and 2% Boehringer Blocking Powder) for 30 minutes, washed with Tris-buffered saline with 0.1% Tween-20, incubated in 2 N HCl for 15 minutes and rinsed four times with 0.1 M sodium borate solution (pH 8.5). After this rinsing, sections were incubated with anti-BrdU antibody overnight and then with secondary Cy3-labeled anti–mouse IgG antibody for 3 hours.

RT-PCR

Total RNA was isolated from E14.5 pancreata using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The isolated RNA was treated with RNAse-free DNAse and then used with the Advantage RT-for-PCR kit (Clontech) and random hexamer primers

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to synthesize cDNA. Two microliters of this reaction product served as a template for PCR using the following primers: *Bsp*-specific forward primer, 5′-ACACTTACCGAGCTTATGAGG-3′ and reverse primer 5′-TTGCGCAGTTAGCAATAGCAC-3′; *Dmp*-specific forward primer, 5′-TGACAATGACTGTCAGGACGG-3′ and reverse primer 5′-GGCTTTGCTACTGTGGAACCT-3′; *Opn*-specific forward primer, 5′-TTCCAAAGAGAGCCAGGAGA-3′ and reverse primer, 5′-TTGGTTACAACGGTGTTTGC-3′.

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References

Bornstein P, Sage EH. Matricellular proteins: extracellular modulators of cell function. Curr Opin Cell Biol. 2002; 14(5):608–16. [PubMed: 12231357]

Brown LF, Berse B, Van de Water L, Papadopoulos-Sergiou A, Perruzzi CA, Manseau EJ, Dvorak HF, Senger DR. Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. Mol Biol Cell. 1992; 3(10):1169–80. [PubMed: 1421573]

Cirulli V, Beattie GM, Klier G, Ellisman M, Ricordi C, Quaranta V, Frasier F, Ishii JK, Hayek A, Salomon DR. Expression and function of alpha(v)beta(3) and alpha(v)beta(5) integrins in the developing pancreas: roles in the adhesion and migration of putative endocrine progenitor cells. J Cell Biol. 2000; 150(6):1445–60. [PubMed: 10995448]

Coppola D, Szabo M, Boulware D, Muraca P, Alsarraj M, Chambers AF, Yeatman TJ. Correlation of osteopontin protein expression and pathological stage across a wide variety of tumor histologies. Clin Cancer Res. 2004; 10:184–90. [PubMed: 14734468]

Denhardt DT, Noda M, O'Regan AW, Pavlin D, Berman JS. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J Clin Invest. 2001; 107(9):1055–61. [PubMed: 11342566]

Edlund H. Pancreatic organogenesis: Developmental mechanisms and implications for therapy. Nat Rev. 2002; 3:524–532.

Falk P, Roth KA, Gordon JI. Lectins are sensitive tools for defining the differentiation programs of mouse gut epithelial cell lineages. Am J Physiol. 1994; 266:G987–1003. [PubMed: 8023947]

Fedarko NS, Jain A, Karadag A, Fisher LW. Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases. FASEB J. 2004; 18(6):734–6. [PubMed: 14766790]

Fierabracci A, Biro PA, Yiangou Y, Mennuni C, Luzzago A, Ludvigsson J, Cortese R, Bottazzo GF. Osteopontin is an autoantigen of the somatostatin cells in human islets: identification by screening random peptide libraries with sera of patients with insulin-dependent diabetes mellitus. Vaccine. 1999; 18(3–4):342–54. [PubMed: 10506661]

Fisher LW, Torchia DA, Fohr B, Young MF, Fedarko NS. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem Biophys Res Commun. 2001; 280(2):460–5. [PubMed: 11162539]

Gravallese EM. Osteopontin: a bridge between bone and the immune system. J Clin Invest. 2003; 112(2):147–9. [PubMed: 12865402]

Gu G, Wells JM, Dombkowski D, Preffer F, Aronow B, Melton DA. Global expression analysis of gene regulatory pathways during endocrine pancreatic development. Development. 2004; 131(1): 165–79. [PubMed: 14660441]

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Habener JF, Stoffers DA. A newly discovered role of transcription factors involved in pancreas development and the pathogenesis of diabetes mellitus. Proc Assoc Am Physicians. 1998; 110(1): 12–21. [PubMed: 9460079]

Kim SK, Hebrok M. Intercellular signals regulating pancreas development and function. Genes Dev. 2001; 15:111–127. [PubMed: 11157769]

Kim JH, Huang Z, Mo R. Gli3 null mice display glandular overgrowth of the developing stomach. Dev Dyn. 2005; 234(4):984–91. [PubMed: 16247775]

Kolb A, Kleeff J, Guweidhi A, Esposito I, Giese NA, Adwan H, Giese T, Buchler MW, Berger MR, Friess H. Osteopontin influences the invasiveness of pancreatic cancer cells and is increased in neoplastic and inflammatory conditions. Cancer Biology & Therapy. 2005; 4(7):740–746. [PubMed: 15970685]

Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM, Hogan BL. Altered wound healing in mice lacking a functional osteopontin gene (spp1). J Clin Invest. 1998; 101(7):1468–78. [PubMed: 9525990]

Mazzali M, Kipari T, Ophascharoensuk V, Wesson JA, Johnson R, Hughes J. Osteopontin--a molecule for all seasons. QJM. 2002; 95(1):3–13. [PubMed: 11834767]

Miyamoto Y, Maitra A, Ghosh B, Zechner U, Argani P, Iacobuzio-Donahue CA, Sriuranpong V, Iso T, Meszoely IM, Wolfe MS, Hruban RH, Ball DW, Schmid RM, Leach SD. Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. Cancer Cell. 2003; 3(6):565–76. [PubMed: 12842085]

Murphy-Ullrich JE. The de-adhesive activity of matricellularproteins: is intermediate cell adhesion an adaptive state? J Clin Invest. 2001; 107(7):785–90. [PubMed: 11285293]

Murtaugh LC, Melton DA. Genes, signals, and lineages in pancreas development. Annu Rev Cell Dev Biol. 2003; 19:71–89. [PubMed: 14570564]

Myers DL, Harmon KJ, Lindner V, Liaw L. Alterations of arterial physiology in osteopontin-null mice. Arterioscler Thromb Vasc Biol. 2003; 23(6):1021–8. [PubMed: 12714436]

Pictet, R.; Rutter, WJ. Development of the embryonic endocrine pancreas. In: Steiner, DF.; Frenkel, N., editors. Handbook of Physiology. Williams and Wilkiins; Washington, D.C: 1972. p. 25-66.

Rittling SR, Chambers AF. Role of osteopontin in tumour progression. Br J Cancer. 2004; 90(10): 1877–81. [PubMed: 15138464]

Slack JMW. Developmental biology of the pancreas. Development. 1995; 121:1569–1580. [PubMed: 7600975]

Suzuki K, Zhu B, Rittling SR, Denhardt DT, Goldberg HA, McCulloch CA, Sodek J. Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion, and resorption in osteoclasts. J Bone Miner Res. 2002; 17(8):1486–97. [PubMed: 12162503]

Wang J, Elghazi L, Parker S, Kizilocak H, Asano M, Sussel L, Sosa-Pineda B. The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic β-cell differentiation. Developmental Biology. 2004; 266(1):178–189. [PubMed: 14729487]

Wilson MJ, Liaw L, Koopman P. Osteopontin and related SIBLING glycoprotein genes are expressed by Sertoli cells during mouse testis development. Dev Dyn. 2005; 233(4):1488–95. [PubMed: 15937924]

Wilson ME, Scheel D, German MS. Gene expression cascades in pancreatic development. Mech Dev. 2003; 120:65–80. [PubMed: 12490297]

Yoshitake H, Rittling SR, Denhardt DT, Noda M. Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. Proc Natl Acad Sci USA. 1999; 96(14):8156–60. [PubMed: 10393964]

Zhu B, Suzuki K, Goldberg HA, Rittling SR, Denhardt DT, McCulloch CA, Sodek J. Osteopontin modulates CD44-dependent chemotaxis of peritoneal macrophages through G-protein-coupled receptors: evidence of a role for an intracellular form of osteopontin. J Cell Physiol. 2004; 198(1): 155–67. [PubMed: 14584055]

Zohar R, Suzuki N, Suzuki K, Arora P, Glogauer M, McCulloch CA, Sodek J. Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. J Cell Physiol. 2000; 184(1):118–30. [PubMed: 10825241]

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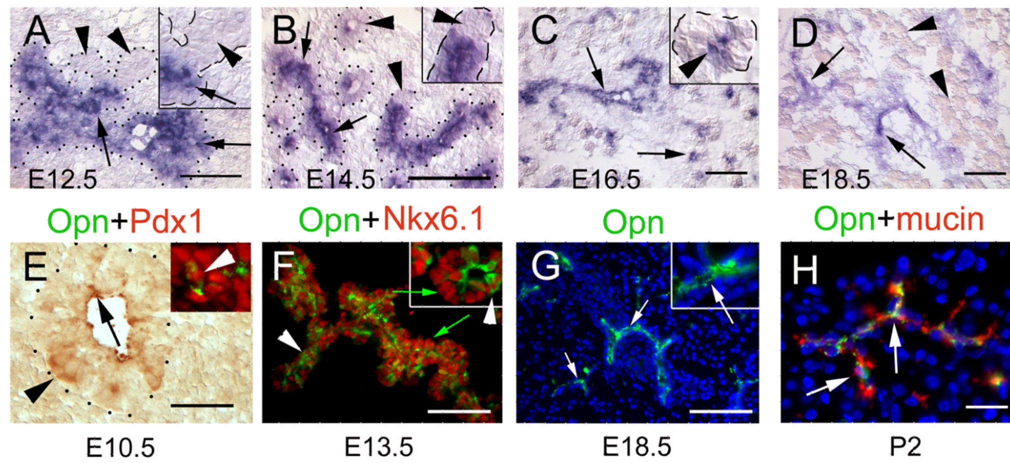

Fig 1.

A–D: *Changes in Opn mRNA Expression Between E12.5 and E18.5. Opn* transcripts (arrows in **A–D**) are broadly distributed in the pancreatic epithelium of embryos dissected between E12.5 (**A**) and E14.5 (**B**), with one exception: Clusters of cells on the basolateral side of epithelial tissue of the pancreas or at the tip of the branches (arrowheads and insets in **A** and **B**) are devoid of *Opn* mRNAs. After E16.5 *Opn* transcripts become restricted to cells in the lumen of the pancreatic epithelium (arrows in **C** and **D**) or to centroacinar cells (arrowhead and inset in **C**). The developing exocrine acini (arrowheads in **D**) are devoid of *Opn* expression. **E–H:** *Changes in Opn protein Expression Between E10.5 and P2*. In developing pancreata, the expression of Opn proteins mimics the pattern of *Opn* mRNAs (shown in **A–D**). At E10.5 (**E**), very low levels of Opn proteins are observed in the cytoplasm of some epithelial cells (arrowheads and green fluorescence in inset) or in the lumen (arrow). At E13.5 (**F**), a considerably increased population of cells expressing Opn immunoreactivity (green fluorescence and arrowheads) appears dispersed throughout the developing epithelium (labeled with anti-Nkx6.1 antibodies, red fluorescence). In these tissues, most Opn^{+} cells express this protein in the cytoplasm (arrowhead in inset), but some epithelial cells are also devoid of Opn immunoreactivity (green arrows and inset). At E18.5 (**G**) Opn proteins (green fluorescence) are largely deposited on the apical side of cells of the developing ducts (arrows and inset). At P2 (**H**), Opn proteins (green fluorescence) co-localize with the ductal marker mucin (red fluorescence) in small ducts (arrows), in large ducts (not shown) and in centroacinar cells (not shown). Insets in **A**, **B**, **C**, **E, F** and **G** are higher magnification images. In **E** (inset), the pancreatic epithelium was labeled with anti-Pdx1 antibodies (red fluorescence). In **G** and **H** the cell nuclei were stained with DAPI (blue). Scale bars: 100 μM (**A–D, F, G**); 200 μM (**E**); 400 μM (**H**).

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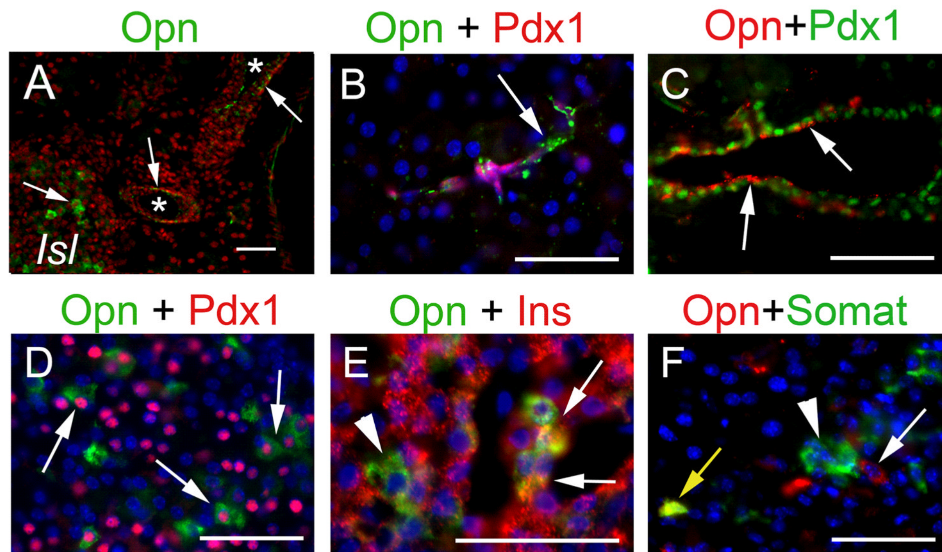

Fig. 2.
Opn is expressed in adult pancreatic tissues of mice. **A**, Low levels of Opn immunoreactivity (green fluorescence) persist in the pancreatic ductal tissue (asterisk) or in the islets (*Isl*) of Langerhans of adult mice. In small intralobular ducts (**B**) or in large ducts (**C**), Opn proteins (arrows; green in **B**, red in **C**) co-localize in cells that also express low levels of the transcription factor Pdx1 (red in **B** or green in **C**). In adult islets (**D–F**), Opn (green in **D**, **E** and red in **F**) also co-localizes with a subset of Pdx1$^+$ cells (arrows in **D**). Some of the Opn$^+$ islet cells also express insulin (arrows in **E**) or, occasionally, somatostatin (yellow arrow in **F**). Arrowheads in **E** and **F** indicate Opn$^+$ cells devoid of insulin (**E**) or somatostatin (**F**) expression. In **B** and **C** the signal of Pdx1 expression in the ducts was enhanced to make this protein more visible. Scale bars: 200 μM.

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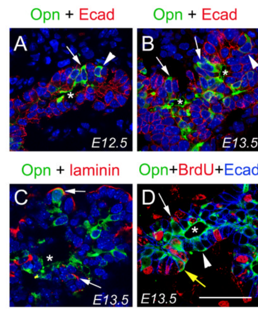

Fig. 3.

In E12.5-E13.5 developing pancreata, numerous cells express Opn proteins in their cytoplasm. Many cells of the E12.5 (**A**) or E13.5 (**B–D**) pancreata expressing cytoplasmic Opn (green fluorescence) appear stretching between the luminal side (asterisks) and the basolateral side of the epithelium (arrows in **A**, **B** and **D**). Some of these Opn^+ cells (arrows in **C**) also appear to be contacting the basal membrane (stained with anti-laminin antibodies, red fluorescence in **C**). Also, some Opn^+ cells (yellow arrow in **D**) are proliferating cells that incorporate BrdU after a 1-hour pulse (red fluorescence; arrowhead indicates an Opn^+/BrdU-negative cell). In **A, B,** and **D**, the pancreatic epithelium was stained with anti-Ecad antibodies (red in **A**, **B**; blue in **D**). In **A–C**, the cell nuclei were stained with TO-PRO-3. All images were taken with a confocal microscope. Scale bar: 200 μM.

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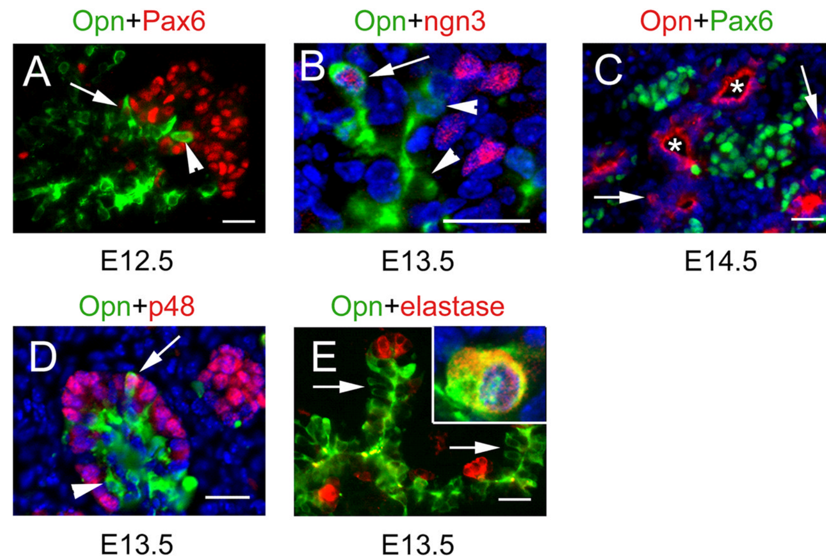

Fig. 4.
Most Opn^+ cells of E12.5-E14.5 pancreatic tissues do not express markers of endocrine or exocrine differentiation. At E12.5 (**A**) and E14.5 (**C**), the vast majority of Opn^+ cells (arrowheads; green fluorescence in **A**, red fluorescence in **C**) do not express the pan-endocrine marker Pax6 (arrow in **A** points to a unique cell co-expressing Opn and very low levels of Pax6). At E13.5 (**B, D**, and **E**) numerous pancreatic epithelial cells express Opn proteins (green fluorescence and arrowheads), but only a few of these Opn^+ cells appear to be co-expressing Ngn3 (an endocrine-specification marker; arrow in **B**), p48 (an exocrine-specification marker; arrow in **D**), or elastase (inset in **E**). Asterisks in **C** denote Opn proteins deposited in the lumen of the pancreatic epithelium. **B** and inset in **E** are confocal images. The cell nuclei were stained with TO-PRO-3 (**B** and inset in **E**) or with DAPI (**C**, **D**). Scale bars: 400 μM.

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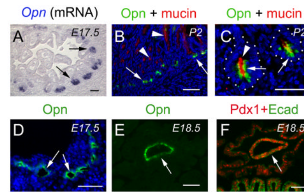

Fig. 5.

Opn is expressed in the stomach and the hepatobiliary system of mouse embryos or newborn mice. *Opn* transcripts (blue staining in **A**) or Opn proteins (green fluorescence in **B** and **C**) are detected in the glandular portion of the stomach of E17.5 embryos (**A**) or newborns (P2; **B** and **C**). In this developing organ, the expression of Opn is particularly abundant at the base of the pits (arrows in **A, B** and dotted outlines in **C**), and in these areas Opn often co-localizes with mucin (red fluorescence in **B**, **C** and arrowhead in **C**). In newborn mice, mucin is also abundantly expressed in the lumen of the stomach (arrowhead in **B**). In the hepatic region of E17.5 embryos (**D**), Opn protein expression (green fluorescence) is detected in the developing intrahepatic bile ducts (arrows), but not in the liver parenchyma (arrowhead). In the duodenum (**E** and **F**) of E18.5 embryos, the expression of Opn (green and arrow in **E**) is detected in the terminal region of the pancreatic duct (visualized in **F** with anti-Pdx1 antibodies, red and arrow) but not in the epithelia of the duodenum (green folds and arrowhead in **F**). **E** and **F** are adjacent sections. In **B–D,** the nuclei were stained with DAPI. In **F,** the epithelium is stained with Ecad antibodies (green fluorescence). Scale bars: 100 μM.

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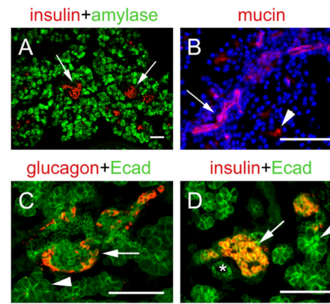

Fig. 6.

The loss of Opn function does not seem to affect pancreas organogenesis. **A**, The pancreata of Opn-deficient newborn mice have a normal distribution of exocrine (green fluorescence) and endocrine (red fluorescence and arrows) tissues. **B**, Such pancreata also have a normal expression of mucin (red fluorescence) in large ducts (arrow) or in centroacinar cells (arrowhead). In addition, in these mutant tissues, the expression of hormones (e.g., glucagon, arrow in **C**, or insulin, arrow in **D**) and the architecture of islets (arrows in **C** and **D**) or acini (arrowheads in **C** and **D**) also seem normal. In **B**, nuclei were stained with DAPI. In **C** and **D**, the epithelia were stained with Ecad antibodies (green fluorescence). Scale bar**s:** 100 0M.

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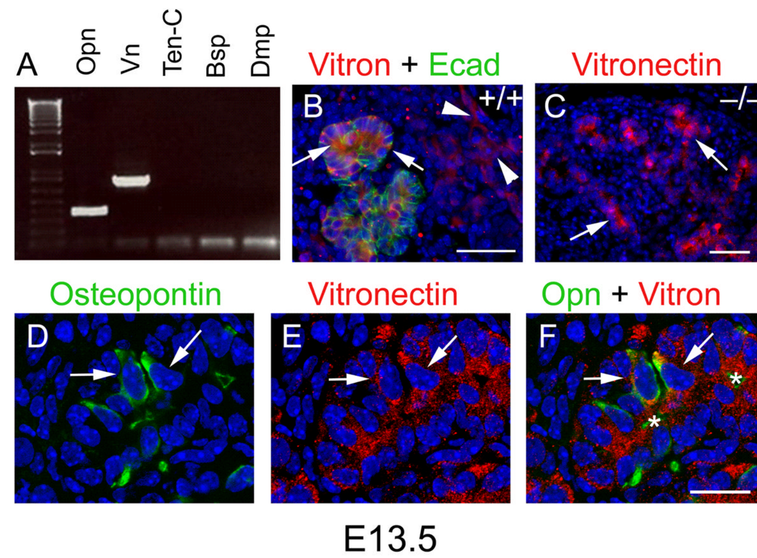

Fig. 7.

In developing pancreata, Opn and Vn are similarly expressed. **A**, Results of RT-PCR analysis of RNA from E14.5 pancreatic tissues show expression of Osteopontin (*Opn*) and Vitronectin (*Vn*) transcripts, but not of those encoding the matricellular protein Tenascin C (*Ten-C*) or the two SIBLING family members *Bsp* or *Dmp*. **B,** In the pancreatic region of E13.5 embryos, expression of Vn proteins (red fluorescence) is detected in the pancreatic epithelium (labeled with green; arrows) and at lower level, in some mesenchymal cells (arrowheads). **C**, Vn (red fluorescence and arrows) is normally expressed in the pancreata of E13.5 Opn-deficient embryos. **D**, At E13.5, expression of Opn proteins (green fluorescence) is detected on the apical side (arrowheads) or in the cytoplasm (arrows) of pancreatic epithelial cells. **E**, In these tissues a large population of epithelial cells also expresses Vn in the cytoplasm (arrows and red fluorescence). **F**, In E13.5 pancreata many Opn^+ cells co-express Vn (arrows), although some Opn^+ cells do not (arrowhead). **D–F**, are confocal images of the same section (**D** and **E** are separate channels and **F** shows the overlapped images). Cell nuclei were stained with DAPI (**B, C**) or with TO-PRO-3 (**D–F**). Asterisks in **F** indicate the lumen of the epithelium. Scale bars: 200 μM.