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ORIGINAL ARTICLE

Epithelial progenitor 1, a novel factor associated with epithelial cell growth and differentiation

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Abstract The growth and renewal of epithelial tissue is a highly orchestrated and tightly regulated process occurring in different tissue types under a variety of circumstances. We have been studying the process of pancreatic regeneration in mice. We have identified a cell surface protein, named EP1, which is expressed on the duct epithelium during pancreatic regeneration. Whereas it is not detected in the pancreas of normal mice, it is found in the intestinal epithelium of normal adult mice, as well as during pancreatic repair following cerulein-induced destruction of the acinar tissue. The distinctive situations in which EP1 is expressed, all of which share in common epithelial cell growth in the gastrointestinal tract, suggest that EP1 is involved in the growth and renewal of epithelial tissues in both the intestine and the pancreas.

Keywords Pancreas · Intestine · Regeneration · SDF1 · CXCR4

Abbreviations

NOD	Non-obese diabetic
SCID	Severe combined immunodeficiency
FACS	Fluorescence activated cell sorting
LCA	Leukocyte common antigen

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SDF1a	Stromal-derived-factor-1 alpha
CXCR4	Chemokine (C-X-C motif) receptor 4
CCK	Cholecystokinin
panCK	Pancytokeratin
BrdU	Bromodeoxyuridine
Ly-6	Lymphocyte antigen 6
uPAR	Urokinase-type plasminogen activator receptor
LU	Ly-6 antigen/uPA receptor-like domain
PLA2	Phospholipase A2

Introduction

The process of epithelial cell growth and renewal is central to the maintenance and repair of a variety of tissue types. In the normal pancreas, expansion of the ductal epithelium is not typically observed [1–3]. However, in situations of pancreatic tissue growth, replenishment, and carcinogenesis, expansion of duct epithelium is an important process. We have studied pancreatic regeneration in mice expressing a transgene encoding IFNg in their pancreatic islets [2–9]. In this model, continuous duct expansion and new islet formation are seen throughout adult life, with endocrine cells arising from duct-localized cells [2, 3]. Extensive proliferation of epithelial cells is observed in the pancreas of the IFNg transgenic mice during regeneration [2, 3]. In addition, the expanding epithelium is associated with beta-cell formation. The involvement of epithelium in pancreatic renewal has been described in other models as well. For example, it has been suggested that tubular duct-like structures, epithelial in nature, might contribute to the development of newly formed acini following cerulein-induced pancreatic damage [10, 11]. In addition, expansion of the ductal epithelium occurs after partial pancreatectomy in rats; in this model, it has been proposed that the newly formed ductules give rise to new endocrine and exocrine tissue [12]. The potential for pancreatic epithelium to give rise to endocrine and exocrine tissues has been reviewed [13–16].

The present studies were undertaken to identify additional molecules that contribute to or promote epithelial cell growth and tissue renewal. In this report, we describe the identification of a protein (which we have named EP1) that is extensively expressed during pancreatic regeneration. In addition, this molecule is also expressed in other situations characterized by epithelial cell expansion, such as in the small intestine and during cerulein-induced acinar tissue damage in the pancreas. Given its tightly regulated expression pattern, we suggest that EP1 could contribute in vivo to the dynamic expansion and renewal of the epithelial compartments in which it is expressed.

Results

Comparison of differentially expressed RNAs between the regenerating and wild-type pancreas

In order to identify factors involved in the extensive regeneration of endocrine cells in the IFNg mouse, we performed subtractive hybridization experiments using RNA from the pancreas of immunodeficient IFNg.SCID and NOD.SCID mice. SCID mice, which lack mature lymphocytes, but which still undergo IFNg-induced pancreatic regeneration, were used to eliminate a contribution to the cDNA pool of sequences deriving from lymphocytes, since IFNg has a myriad of effects on such populations, many of which would be reflected in our cDNA profile analysis. Through these analyses, we identified and isolated a cDNA sequence (epithelial progenitor 1, EP1, GenBank accession number AY148487) whose expression is significantly enhanced in the IFNg pancreas, as determined initially through Northern blot analysis (data not shown), and later confirmed and characterized in greater detail using in situ hybridization and immunohistochemistry (Fig. 1a–d, described in more detail later).

Characterization of EP1 protein expression during pancreatic regeneration

We developed monoclonal antibodies against EP1 to analyze its expression patterns in various tissues. Whereas EP1 was not detected at any embryonic stage, neither in wild-

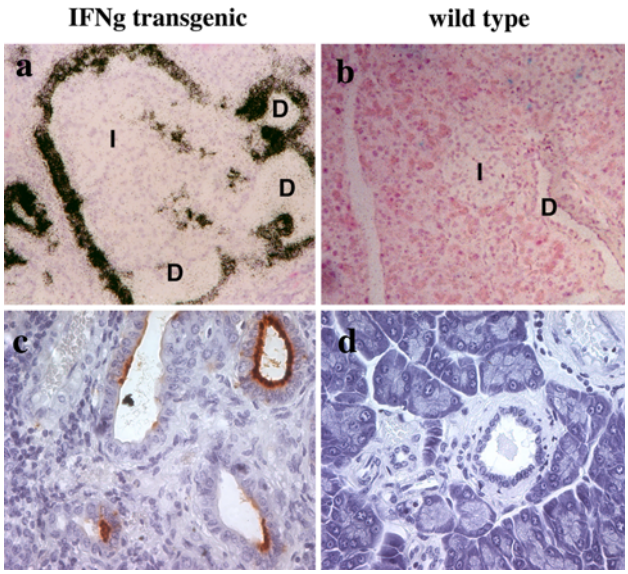

Fig. 1 Pancreatic expression of EP1. Pancreatic sections from IFNg transgenic (**a**, **c**) and non-transgenic (**b**, **d**) mice were examined by in situ hybridization (**a**, **b**) or immunohistochemistry (**c**, **d**) for the detection of EP1 mRNA or protein, respectively. *I* islets, *D* ducts. Original magnification: ×40

type nor IFNg transgenic animals, strong expression of EP1 was observed in the pancreas of adult animals. In agreement with our earlier in situ hybridization analysis, EP1 was detected in the ducts of the IFNg regenerating pancreas, but was not found in the islets or acinar tissue (Fig. 1c). The EP1 staining was not uniform in all of the duct cells, but was extremely focal in nature. We found regions of large ducts as well as many smaller ducts that stained intensely. The staining pattern was highly localized, with the EP1 antigen concentrated on the lumenal surface of the polarized duct epithelial cells. Furthermore, EP1 staining was not observed in the pancreatic tissue from control non-transgenic littermates (Fig. 1d). We also performed additional control experiments to investigate EP1 expression in transgenic mice expressing other cytokines in their beta cells, including IL-4, IL-10, or TGF-b. These molecules are known to affect the immune system, generating inflammation without stimulating beta-cell growth [17, 18]. However, pancreatic expression of EP1 was specific to the IFNg mouse, as EP1 staining was not observed in the pancreas of other cytokine transgenic mice (data not shown).

In order to determine the pattern of EP1 expression postnatally and into adulthood, we studied the pancreas of transgenic mice from birth to 6 months of age. In the IFNg transgenic mouse, duct cell expansion associated with endocrine cell formation progresses with age. Interestingly, increased EP1 expression was found to coincide with expansion of the ductal network. We observed only rare EP1-expressing cells prior to 2 weeks of age. However, cells expressing the EP1 antigen could be observed more frequently by 2 weeks of age. The cells were found to expand in numbers as the mice aged, and by 4 months of age, they represented approximately 5–10% of the duct epithelial cells in the transgenic mouse pancreas (data not shown). These data indicate that EP1-expressing cells expand during adult life in the transgenic mice, concomitant with pancreatic regeneration.

Expression of EP1 in the gut epithelium of normal adult mice

Our initial Northern blot analysis revealed that EP1 is also copiously expressed in the normal adult stomach and intestine (data not shown). Using immunohistochemistry to localize the EP1 antigen, we found that the epithelium of the stomach and intestine stained strongly with EP1 antibody (Fig. 2a and data not shown). Expression levels were consistent from birth in these tissues, with EP1 expressed throughout adulthood. In the intestine, the staining was located on the lumen of the intestinal villi, starting at the base of the villi and continuing along the entire length. Staining for EP1 was not typically observed in the crypt region of the intestinal villi (Fig. 2a). Furthermore, staining was greatest in the duodenum and tapered off distal to this region in the small intestine; EP1 expression was not observed in the large intestine. We also found substantial expression of EP1 in the adult intestine using FACS analysis, gating on the CD45 LCA negative population, with approximately 20–30% of cells prepared from the small intestine positive for both the epithelial cell marker CD49f and the EP1 protein (Fig. 2b).

EP1-positive cells express receptors for the critical chemotactic factor SDF1a

The above-described expression patterns of EP1 suggest that this protein might participate in the continuous renewal of epithelial cells during pancreatic regeneration in the IFNg mouse. We have previously demonstrated that the SDF1a/CXCR4 chemokine/chemokine receptor axis is important in the expansion of duct cells during pancreatic regeneration, with inhibition of this axis significantly reducing duct cell proliferation [5]. Therefore, we asked if EP1-expressing cells in the IFNg pancreas also express CXCR4. Indeed, FACS analysis of isolated pancreatic duct cells from IFNg mice demonstrated that a substantial portion of the EP1-positive cells also expressed CXCR4, suggesting that this cell population could respond to SDF1a during pancreatic regeneration (Fig. 3). Given this, we sought to determine if epithelial cell expression of EP1 could be induced by pancreatic expression of SDF1a. We obtained transgenic mice that express SDF1a under control of the rat insulin promoter from Dr. Jason Cyster, University of California, San Francisco [19], and we screened these mice for the presence of the EP1 antigen in the pancreas. However, whereas EP1 was present in the intestinal tract of these mice, it was not detected in the pancreas of the SDF1a-expressing mice (data not shown).

SDF1a and pancreatic damage promote EP1 expression in the pancreas

We next sought to determine if EP1 expression could be induced in response to pancreas damage, independent of overexpressed IFNg. For these analyses, we treated C57BL/6 mice with the CCK analog cerulein, which damages exocrine pancreatic tissue, while sparing the ducts and islets. While the damage and necrosis wrought by cerulein can be extensive, it is not permanent, and the exocrine pancreas can repair itself relatively quickly [20, 21]. The mechanism of acinar tissue regeneration likely involves several processes, the most productive being the proliferation of newly formed acinar cells [11, 21]. In addition, the tubular duct-like structures present after

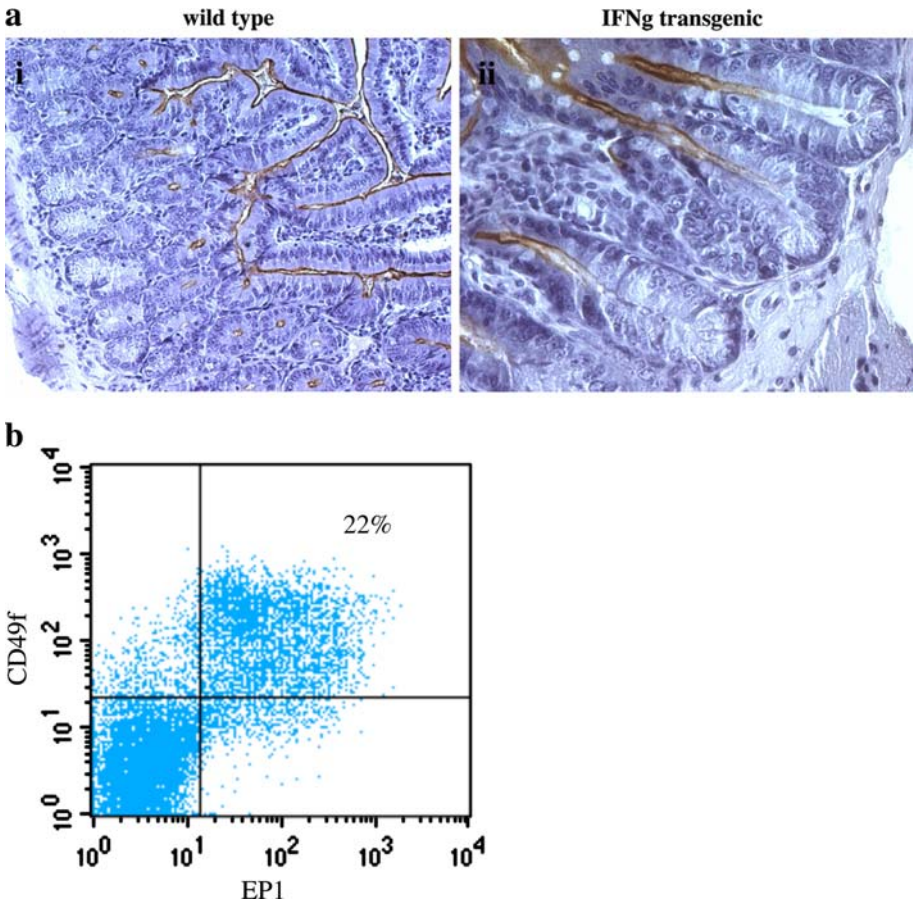

Fig. 2 **a** Intestinal expression of EP1. Tissue sections from the small intestines of wild-type (i) and IFNg transgenic (ii) mice were stained with antibody against EP1. Original magnification: panel (i), ×20; panel (ii), ×40. **b** FACS analysis of EP1 in intestinal epithelium. Intestinal epithelial cells were harvested from IFNg transgenic mice and stained with antibody against EP1 for FACS analysis. As indicated by the dot plot, 22% of the cells in the intestinal preparation (gated on the CD45 LCA negative population) were EP1 positive epithelial (CD49f positive) cells

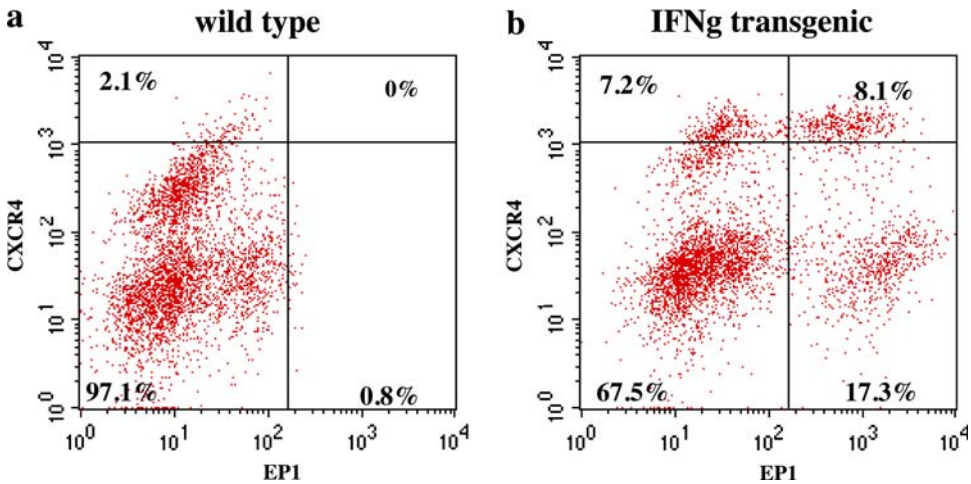

Fig. 3 FACS analysis of EP1 and CXCR4 expression in pancreatic epithelial cells isolated from wild-type (**a**) or IFNg transgenic (**b**) mice. Four mice were analyzed per group, and the data shown in each panel, gated on the CD45 LCA negative/CD49f positive population, is representative of one individual mouse per group

cerulein-induced damage are also thought to contribute to acinar cell regeneration. These structures are epithelial in nature (panCK-positive; Fig. 4a). They are thought to arise either from pre-existing ductal structures [11], or through the re-differentiation or de-differentiation of acinar cells to duct-like cells [10, 22–24]. For these studies, we screened cerulein-treated mice for the presence of EP1 after a two-week course of treatment with cerulein (see "Materials and methods"). However, we did not observe any EP1 expression in the pancreas of these mice (Fig. 4b). In contrast, when we treated the SDF1a transgenic mice with cerulein for 2 weeks, EP1 expression was observed in scattered epithelial structures throughout the pancreas (Fig. 4c, d). These EP1-expressing cells included those in the centroacinar ducts, as well as those in the tubular ducts that are associated with acinar–ductal transitional

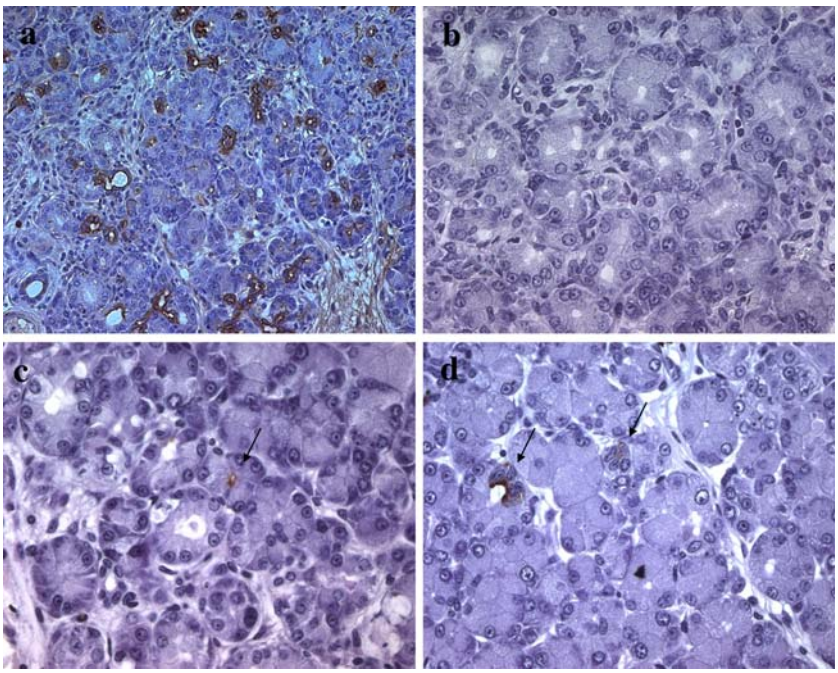

Fig. 4 **a** panCK staining of the cerulein-treated NOD pancreas highlights the epithelial nature of the tubular structures that characterize the repair response. **b–d** EP1 expression in the pancreas of cerulein-treated wild-type mice (**b**), and cerulein-treated SDF transgenic mice (**c**, **d**). EP1 expression is indicated by the arrows in panels c and d. Original magnification: ×40

structures. Although, the EP1-expressing cells were relatively rare, as indicated by the representative field shown in Fig. 4, the results were consistently observed in three separate experiments. That is, EP1 expression was never observed in the pancreas of C57BL/6 mice, with or without cerulein treatment, but was always observed, in very small numbers, in SDF1a transgenic mice treated with cerulein. While no discernable difference in the repair process was evident between the cerulein-treated SDF1a transgenic and normal mice, these data provide a second model of pancreatic re-growth in which the EP1 protein is induced during tissue renewal. Importantly, the IFNg and SDF1a/cerulein models share in common the expression of EP1 specifically during the expansion of epithelial cells.

Mitotic activity of EP1-expressing epithelial cells

The IFNg pancreas, which exhibits continuous epithelial cell expansion, is characterized by a high degree of mitotic activity [2]. Given their presence under conditions of tissue growth and renewal, we sought to determine if EP1-expressing cells are mitotically active in vivo. For this purpose, we performed FACS analysis of intestinal epithelial cells from BrdU fed animals. Our results demonstrated that approximately 75% of the EP1-positive epithelial cells in the intestinal epithelium (identified as CD49f positive and CD45 negative) are also BrdU positive (data not shown), indicating that the majority of EP1-expressing intestinal epithelial cells are mitotically active.

Discussion

The identification of factors involved in epithelial tissue renewal will be important if approaches to promote or facilitate this process under circumstances of tissue insufficiency are to be devised. We have identified a cell surface protein that is expressed on epithelial cells in the pancreas and intestine during growth and repair processes, which we have designated as EP1.

The intestine is composed of a myriad of epithelial folds (villi), which continue to grow and turnover from the embryonic stages throughout adult life [25, 26]. In contrast, duct expansion is minimal in the normal pancreas, with most ducts limited in size and exhibiting little proliferation throughout adult life. This is in contrast to the IFNg transgenic pancreas, where continuous duct expansion occurs throughout adult life [2]. Interestingly, although it was highly expressed in the ductal epithelium of the IFNg transgenic pancreas, we did not observe EP1 expression in the fetal pancreas (data not shown) or in the wild-type adult pancreas at any stage. Thus, the normally occurring events related to pancreatic development and duct formation appear to be independent of EP1. In contrast, we observed strong EP1 expression in the gastrointestinal epithelium throughout adulthood, as well as during embryogenesis. These data suggest that EP1 is involved in the process of epithelial cell expansion in the gut from an early stage, and that it continues to remain involved in this process during adult life. These data are consistent with the presence of EP1 in tissues exhibiting continuous epithelial cell growth,

as in the IFNg transgenic and cerulein-treated mouse models described in this report.

In the present study, we report that EP1-expressing cells are observed in the cerulein-treated pancreas if SDF1a is also present. Cerulein is a CCK analog that destroys cells in the exocrine tissue [27, 28]. However, despite the potency of this chemical, the damage is completely reversible, with newly formed and functional acini arising in the damaged tissue relatively quickly [20, 21, 29]. These acini are believed to be formed through a combination of processes, including enhanced replication of pre-existing acinar cells, as well as their generation from epithelial structures (tubular ducts) that arise throughout the acinar tissue [10, 11, 21]. In the absence of damage, with or without SDF1a, EP1 is not observed in the pancreas. Furthermore, in the absence of SDF1a, the cerulein-induced damage is completely repaired, but EP1 is not found. Clearly, EP1 is not needed for repair of cerulein-induced pancreatic damage. However, when SDF1a is present during cerulein treatment, EP1 is observed in a small portion of the centroacinar cells and tubular ducts. Therefore, a strong link between the SDF1a/CXCR4 axis and the presence of EP1-expressing cells exists, as it is only in the presence of both SDF1a and tissue damage/renewal that EP1 is found in the (normal) pancreas.

Interestingly, the SDF1a/CXCR4 axis is involved in epithelial cell migration and responsiveness in both the normal intestine and the IFNg transgenic/regenerating pancreas. For example, numerous excellent studies have demonstrated that SDF1a/CXCR4 are expressed in intestinal epithelial cells in humans and rats [30–32]. This axis has been shown to direct intestinal epithelial cell migration as well, promoting maturation and repair of the mucosal barrier [32, 33]. In addition, our previous studies have also shown that SDF1a/CXCR4 ligation is important for epithelial cell migration and expansion in the IFNg transgenic pancreas [5]. Furthermore, we report here that EP1-expressing cells in the regenerating pancreas also express the SDF1a receptor, CXCR4, which would enable them to respond to SDF1a and migrate as this signal dictates. These data suggest that the expansion of EP1-expressing cell populations and SDF1a-facilitated cell migration and movement through the matrix during tissue renewal are intimately coupled.

How might EP1 facilitate the expansion of epithelium in growing and renewing tissues? Interestingly, sequence analysis has indicated that EP1 is related to the uPAR/CD59/Ly-6/snake toxin family. This family is composed of cysteine-rich proteins, containing "LU" domains of approximately 90 amino acids with up to 10 cysteines that form internal disulfide bonds [34–38]. The family member with which EP1 is most homologous, sharing 42% identity and 60% homology, is rodent bone 1 (RoBo-1; Fig. 5). RoBo-1 is expressed specifically in the lengthening growth plate of the bone, where cartilage is remodeled into bone, and is upregulated by modulators of bone metabolism [39]. Interestingly, EP1 and RoBo-1 have several important features in common. For example, whereas most of the uPAR/CD59/Ly-6/snake toxin family members so far identified have one cysteine-rich domain (other than uPAR, which has three such domains), only a few family members, including EP1 and RoBo-1, as well as the metastasis-associated antigen C4.4 from rat [40, 41] and the secreted PLA2 inhibitor from cobra blood [42], have two such domains. In addition, both EP1 and RoBo-1 exhibit highly restricted expression patterns, with upregulation evident in specific situations: pancreatic regeneration and intestinal renewal for EP1 and after stimulation of bone formation for RoBo-1 [39]. The similarities in their sequences, structures, and induced expression patterns suggest that they might serve related functional roles as well, facilitating the expansion of their target cell populations under conditions of tissue growth, renewal, or repair.

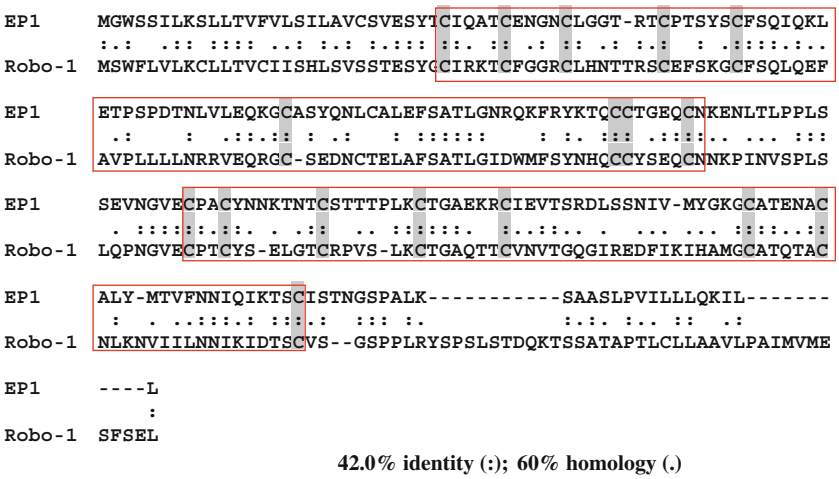

Fig. 5 Sequence homology between the EP1 protein coding sequence and that of RoBo-1. The LU domains are outlined in red

Interestingly, a cDNA sequence identical to EP1 was submitted as part of a GenBank submission from the NIH-Mammalian Gene Collection Project (accession number BC019553); this cDNA was isolated from a biopsy sample of a mammary tumor, suggesting that EP1 might serve a more global role in epithelial cell expansion, such as during the growth of malignancies. Sequences originating in thymus, heart, and spleen have also been deposited. This is the first report on EP1 mRNA and protein expression. In build 37.1 of the Mus musculus genome, the gene encoding EP1 (official symbol: 2210407C18Rik) maps to chromosome 11 (location 11 B1.3) and consists of six exons and spans slightly over 5 kb.

In conclusion, our data suggest that EP1 is involved in tissue renewal in the regenerating pancreas as well as in the normal intestine. Its additional presence under circumstances of tissue repair after cerulein-induced tissue damage, dependent on SDF1a, and during mammary gland tumorigenesis (reflected by the isolation of its cDNA from a mammary tumor), suggests that it plays a more global role in epithelial cell expansion. Indeed, while it is clear that a variety of factors and signals promote and direct the formation of new endocrine cells, our data suggest that one such signal emanates from the EP1 protein. The identification of this tightly regulated epithelial cell surface molecule could be important for the development of targeted therapeutics aimed at promoting or limiting epithelial cell expansion under different disease conditions, as well as for studies investigating beta-cell formation or expansion.

Materials and methods

Animal husbandry and transgenic mice

Animals were maintained in a specific pathogen-free facility at The Scripps Research Institute according to the rules and regulations governed and enforced by the Institutional Animal Care and Use Committee. Animals were housed under a controlled 12-h light/dark cycle and provided with food and water ad libitum. For in vivo labeling of mitotically active intestinal and pancreatic epithelial cells, BrdU (Sigma-Aldrich, St. Louis, MO, USA) at 80 mg/100 ml was added to the drinking water; water bottles were changed daily. After 8 days, the mice were killed and the intestinal or pancreatic epithelial cells isolated and analyzed by FACS as described below.

Subtractive hybridization

Suppressive subtractive hybridization was performed to isolate novel cDNA sequences characterizing pancreatic regeneration [43]. For this purpose, pancreatic RNA from IFNg.SCID and non-transgenic SCID mice was used to prepare cDNA for the analysis. Total RNA from the pancreas was prepared after mechanical homogenization in solution D (4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol), followed by passage through a 18–21 gauge needle three times to shear genomic DNA. After the addition of 0.1 volume 2 M NaOAc pH 4.0, 1 volume phenol, and 0.2 volumes chloroform, samples were vortexed and placed on ice for 15 min, after which they were centrifuged at 10,000 rpm for 15 min at 4°C. The aqueous phase was then removed, mixed with an equal volume of isopropanol, and incubated for 1 h at −20° C to precipitate the RNA. The RNA was pelleted by centrifugation at 10,000 rpm for 15 min at 4°C, after which the RNA was re-precipitated by dissolving in 500 µl solution D and adding 500 µl isopropanol. The RNA was recovered by centrifugation, washed once with 70% ethanol, and dissolved in 0.1% SDS and 1 mM EDTA, pH 8.0. PolyA+ RNA was isolated from the total RNA using the Micro-FastTrack kit from Invitrogen, San Diego, CA.

For subtractive hybridization, the PCR Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) was used. Briefly, two portions of cDNA synthesized from the IFNg/NOD.SCID polyA+ RNA were ligated in separate reactions with two different adapter sequences. These two sets of adapter-ligated IFNg/NOD.SCID cDNA samples were then separately hybridized to NOD.SCID cDNA. Next, these two hybridization samples were mixed and a second hybridization was carried out, allowing the differentially expressed cDNAs to form hybrids, which were then used as templates for PCR amplification using primers specific for the ligated adapters. The PCR was performed using a Mini-Cycler (MJ Research, Watertown, MA, USA). Agarose gel electrophoresis was used to separate the amplified cDNA molecules; different molecular size pools were purified from the gel, ligated into the pZero-1.1 vector (Invitrogen, San Diego, CA, USA), and transformed into bacteria. Numerous cDNA clones that were potentially differentially expressed resulted from the suppressive subtractive hybridization screening. The inserts of the recombinant plasmids from the resulting cDNA clones were isolated and used as probes in slot-blot analysis to identify those whose inserts hybridized more intensely to IFNg/NOD.SCID total RNA than to NOD.SCID total RNA. Zeta-probe GT Blotting Membranes (BioRad, Hercules, CA, USA) were used for this purpose, along with α^{32}P-dCTP-labeled probes prepared by random-prime labeling of the subtracted IFNg/NOD.SCID cDNA clone inserts. These studies were followed by Northern blot analysis of candidate clones, through which the EP1 cDNA was identified as a clone that was highly expressed in the IFNg, but not

non-transgenic pancreas. We submitted the EP1 sequence to GenBank in 2002 (accession number AY148487).

In situ hybridization

In situ hybridization was performed to characterize the EP1 expression pattern in detail. EP1-containing plasmid DNA was linearized and purified using Proteinase K digestion, phenol extraction, and ethanol precipitation. RNA was transcribed and radioactively labeled with ^{35}S using the linearized DNA as template and Stratagene kit # 200340 (Stratagene, La Jolla, CA, USA). The labeled RNA probe was used in hybridization analyses with deparaffinized, hydrated, protease-treated sections of the IFNg transgenic and non-transgenic pancreas. Superfrost Plus slides were used during these procedures. Slides were prehybridized for 3 h at 46°C in HB2 buffer [50% formamide, 0.3 M NaCl, 20 mM Tris pH 8, 5 mM EDTA, 1× Denhardt's (0.02% acetylated bovine serum albumin, 0.02% Ficoll, and 0.02% PVP-90 (polyvinyl-pyrrolidone)), 10 mM dithiothreitol (DTT), and 10% Dextran sulfate]. Hybridization with the radiolabeled anti-sense EP1 probe was carried out in HB2 buffer overnight at 46°C in a tightly sealed box. Control reactions were performed in parallel using radiolabeled sense EP1 probe. Following hybridization overnight, the slides were washed twice in 2× SSC (0.3 M NaCl, 0.03 M NaCitrate, pH 7.0)/β-mercaptoethanol/EDTA at room temperature, treated with RNAse A for 30 min at room temperature, and washed three times for 1 h per wash in 0.2× SSC/β-mercaptoethanol/EDTA at 42°C. Slides were dehydrated, dried, and exposed to film for 2–5 days.

Anti-EP1 monoclonal antibody generation

The decoy method, described in Yin et al. (1997), was utilized for the generation of monoclonal antibodies against EP1 [44]. Retroviral-mediated transduction of the EP1 cDNA coding sequences into rat NRK cells was followed by the immunization of Dewey-Sprague rats with EP1-transduced or non-transduced control cells. Approximately 3–5 $\times$ 10^5 transduced cells in PBS, pH 7.4, were injected into the right-hand footpad on days 0, 3, 6, 9, and 13; an equal number of control cells were injected into the left-hand footpad on days −3, 0, 6, 9, and 13. Following this two-week immunization schedule, the right-hand popliteal lymph nodes were harvested from the site of injection. A lymphocyte suspension from the lymph nodes was used for cell fusions to SP2/0 Ag14 mouse myeloma cells for hybridoma generation. Hybridoma supernatants were screened for EP1-reactivity on cytospins of transfected EP1-expressing cells and on pancreatic sections from IFNg transgenic mice. A number of clones that reacted with the EP1-expressing transfected cells and that stained tissue from the IFNg pancreas with an identical specificity to the in situ hybridization probe were identified. Several of these hybridoma clones were further purified and expanded and their antibody was isolated. Further characterization of the staining characteristics on tissue revealed that one of the antibodies (clone 14D4) worked well on Bouin's fixed tissue and, less intensively, on formalin fixed tissue. This clone, which worked well in FACS studies as well, was used for subsequent histological analysis.

Immunohistochemistry

Pancreata were fixed for 4 h in Bouin's fixative and embedded in paraffin. Four micrometer paraffin sections were either conventionally stained with hematoxylin and eosin or were stained with antibody using standard immunohistochemical techniques. Tissue sections were deparaffinized and blocked with 10% normal goat serum before application of the primary antibody (rat anti-EP1 clone 14D4, 1:50; panCK (Sigma-Aldrich, St. Louis, MO, USA), 1:100; guinea pig anti-insulin (DAKO, Carpinteria, CA, USA), 1:15,000; rat anti-BrDu (Accurate Chemical, Westbury, NY, USA). Binding of the primary antibody was detected using goat anti-rat (EP1 and BrdU) or goat anti-guinea pig (insulin) secondary antibody (Vector Laboratories, Burlingame, CA, USA), and the horseradish peroxidase (HRP)-labeled avidin–biotin complex (ABC kit, Vector Labs, Burlingame, CA, USA). HRP was visualized using 3,3′-diaminobenzidine as a substrate. Gill's hematoxylin was used as a counterstain.

Cell isolations and FACS analysis

Intestinal epithelial cells were prepared using a modification of the protocol detailed in Current Protocols in Immunology [45]. For this study, the small intestine was dissected out (from the stomach to the large intestine), after which the intestinal contents were pressed out. The intestine was rinsed with HBSS (Hank's Buffered Salt Solution)/5% FCS, cut longitudinally, and then cut into approximately 0.5 cm pieces. Intestine pieces were washed with HBSS/5% FCS, and then incubated in HBSS/5% FCS + 0.15 mg/ml dithioerythritol (DTE; Sigma–Aldrich, St. Louis, MO, USA). The samples were gently shaken at 37°C for 30 min, and the media removed, after which they were washed twice with HBSS/5% FCS + 0.15 mg/ml DTE. The intestinal pieces were allowed to settle, after which the supernatant was collected, pelleted, and the cells resuspended in 25 ml PBS. A total of 50 µl of Dispase (5 mg/ml) was added, and the samples were incubated for 20 min at 4°C. Cells were

then pelleted, resuspended HBSS/5% FCS, and filtered through a 70 μm cell strainer. After pelleting the cells one final time, they were resuspended in FACS buffer (see below) and counted.

Pancreatic epithelial cells were isolated as follows: the whole pancreas was digested in 1 mg/ml collagenase P in DMEM/F-12, with shaking at 37°C. After 30 min, the samples were checked for the appearance of the ductal network, and thereafter every 5 min if necessary. The samples were mixed gently and filtered over a 200 μm nylon mesh. The material on the mesh was reserved and rinsed off with cold PBS. The samples were pelleted and 2 ml 0.05% trypsin/EDTA was added to each. After gentle mixing, the samples were incubated for 15 min at 37°C. The trypsin was then neutralized with an equal amount of FCS, and the samples were filtered over a 70 μm cell strainer. After one final wash with PBS, the cells were pelleted and resuspended to count.

For FACS staining, 5×10^5 cells in 100 μl were stained with 0.5 μg EP1, CD45, CD49f, or CXCR4 monoclonal antibodies. The EP1 antibody was generated as described above, and the CD45, CD49f, and CXCR4 antibodies were from Pharmingen/BD Biosciences (San Jose, CA, USA). Samples were incubated for 20 min on ice, and were washed with 3 ml wash buffer (0.5% BSA/0.01% NaN_3 in PBS). When biotin-labeled EP1 was used, 0.5 μg Streptavidin labeled with a fluorochrome was added. When FITC conjugated antibody was used, samples were fixed with 1% paraformaldehyde. 7-AAD was added to determine the viability of the samples.

For BrdU staining and FACS analysis, we followed the protocol provided with the BrdU Flow kit by BD Pharmingen (San Diego, CA, USA). Briefly, 5×10^5 cells in 100 μl were fixed and permeabilized with BD Cytofix/Cytoperm buffer, treated with DNAse to expose incorporated BrdU, and stained with fluorescent anti-BrdU antibody. A BD FACS Calibur was used to acquire the data, which was subsequently analyzed using CellQuest software. Appropriate isotype controls were used to determine the background staining.

Cerulein treatment

Cerulein (Sigma-Aldrich) in PBS was administered to male mice (6–8 weeks of age) at 200 mg/kg i.p. every hour for 6 h, thrice a week for 2 weeks. Equivalent numbers of age-matched littermates were injected with comparable volumes of PBS in parallel control studies. The mice were killed for histological analysis at the end of the course of cerulein treatment. The experiment was performed three times with 3–4 mice per treatment group in each experiment.

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