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Epithelial-mesenchymal interactions are critical for normal pancreas development. Fibroblast growth factor (Fgf)-10 is expressed in the pancreatic mesenchyme and its signalling is required for normal growth and regulation of gene expression in the pancreatic epithelium. However, little is known about putative Fgf signalling to the mesenchyme. Here we have examined the embryonic pancreas expression of differentially spliced Fgf receptor isoforms and their targets; the Sprouty (Spry) and Spred family genes which are induced by Fgf signalling. Using qPCR to quantify mRNA levels in microdissected pancreatic epithelium and mesenchyme as well as in FACS isolated Pdx1-GFP+ and -GFP− cell populations we demonstrate that several members of the Sprend and Sprouty families are expressed in embryonic mouse pancreas and find Spred1 and -2 as well as Spry2 and -4 to be predominantly expressed in pancreatic mesenchyme. Using embryonic pancreas explant cultures we demonstrate that Spred1/2 and Spry2/4 expression is regulated by Fgf receptor signalling and is increased by treatment with Fgf9, but not by Fgf7 or Fgf10. We extend previous work showing that Fgf9 is expressed in pancreatic mesenchyme, and since Fgf9 is known to activate the mesenchyme-specific "c"-splice forms of Fgf receptors, while Fgf7 and -10 both activate the epithelium-specific "b"-splice forms of Fgf receptors, these results suggest that Fgf signalling is active in the pancreatic mesenchyme, where expression of Spred1/2 and Spry2/4 appear downstream of Fgf9 signalling.

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alternatively spliced at exon 3, encoding the D3 domain region of the receptor, giving rise to one of two alternative “b” or “c”-isoforms (Itoh and Ornitz, 2004). The “b” isoforms are typically expressed in epithelia, while the “c” isoforms are commonly found in mesenchymal tissues, forming the basis for specificity during reciprocal epitheliomesenchymal signalling (Ornitz and Itoh, 2001; Ornitz et al., 1996; Zhang et al., 2006).

As mentioned above, numerous studies have documented the role of Fgf7 and 10 in the regulation of the pancreatic epithelium, but little is known about Fgf signalling to the pancreas mesenchyme. In zebrafish, Fgf24 signalling from pancreatic endoderm to lateral plate mesoderm is required for ventral pancreas development (Manfroid et al., 2007), but as Fgf24 is not conserved in mammals it is unclear if and how mesenchymal Fgf signalling is achieved in these species. To begin to understand Fgf signalling in pancreas mesenchyme we analyzed the expression of FGFR isoforms and downstream target genes of FGFR signalling from the Sprouty- and Sprd-families in isolated pancreatic epithelial and mesenchymal tissue preparations. We demonstrate epithelium-specific expression of FGFR1b, -2b, and -4 and mesenchymal-specific expression of FGFR1c and -2c in E11.5 and E13.5 mouse pancreas. Notably, we find Sprd1 and -2 as well as Spry2 and -4 transcripts enriched in the mesenchymal fraction, and explant studies demonstrate that pancreatic Sprd and Sprouty expression is regulated by FGFR signalling. Additionally, treatment of pancreatic explants with Fgf2, -4, -7, -9, and -10 show that only those Fgfs that efficiently activate the FGFR “c” isoforms (Ornitz et al., 1996; Zhang et al., 2006) can also stimulate Sprd and Sprouty expression.

As we confirm and extend previous observations on expression of Fgf9 in pancreatic mesenchyme, our results suggest collectively that Fgf9 signalling from the outer mesothelial lining induce Sprd/Sprouty expression in the underlying pancreatic mesenchyme.

1. Results

1.1. Embryonic mouse pancreas mesenchyme express FGF receptor Iic splice forms and Fgf9

To begin to understand the potential for mesenchymal Fgf signalling we first analyzed the expression of FGFR splice forms and candidate Fgfs in embryonic mouse pancreas epithelium and mesenchyme by qPCR. We separated E11.5 dorsal pancreatic epithelia from their surrounding mesenchyme and isolated RNA from both fractions, which was subsequently processed for qPCR analysis. To determine the purity of each fraction we analyzed the expression of epithelium-specific markers (Cdh1 (E-cadherin), Foxa2, Glucagon and Pdx1) or of mesenchymal markers (Hgf, Vim (vimentin), and Flk1). As expected, we found mRNA for known epithelial markers to be

![Fig. 1. Expression of FGFR splice forms and selected Fgfs in the E11.5 mouse embryonic pancreas. (A) qPCR analysis of known epithelial and mesenchymal markers in isolated epithelium and mesenchyme. Vimentin, Flk1 and HGF were used as mesenchymal markers and E-cadherin, Foxa2, Glucagon and Pdx1 were used as epithelial markers. (B), qPCR analysis for FGFR splice form expression in isolated epithelium and mesenchyme. (C) qPCR analysis of Fgf9 and Fgf10 expression in isolated epithelium and mesenchyme. Note that both are found enriched in the mesenchyme. Data represent mean ± SEM (n = 3); *p < 0.05, **p < 0.01.](image-url)
abundant in the epithelial fraction but scarce in the mesenchymal fraction (Fig. 1A), suggesting that our mesenchymal fraction is relatively pure. Conversely, we found known mesenchymal markers to be abundant in the mesenchymal fraction as expected, but also detected lower levels of these in the epithelial fraction, indicating that the latter is contaminated by mesenchymal cells (Fig. 1A). We next examined the expression of the different FGFR splice forms in the epithelial and mesenchymal fractions. As expected we found mRNA for the known epithelial receptor isoforms FGFR2b and FGFR4 (Elghazi et al., 2002) to be enriched in our epithelial fraction, while other putative epithelial specific isoforms (FGFR1b and -3b) were only expressed at low levels (Fig. 1B). FGFR1 has previously been found in both pancreatic epithelium and mesenchyme (Elghazi et al., 2002) but no distinction was made between "b"- and "c"-splice forms. In the epithelial fraction we found low levels of FGFR1b, slightly enriched compared to the mesenchymal fraction (Fig. 1B) However, in the mesenchymal fraction we found enrichment for mRNA encoding FGFR1c as well as -2c and the expression levels were comparable to the epithelial FGFR2b and FGFR4 (Fig. 1B), suggesting that these receptors may mediate mesenchymal Fgf signalling. Expression of FGFR3 splice forms has to our knowledge not previously been analyzed in isolated epithelial and mesenchymal fractions from the pancreas. We found low levels of FGFR3b and ~10-fold higher levels of FGFR3c. However, we found no enrichment of either splice form in the epithelial or mesenchymal fractions (Fig. 1B). Among the Fgf ligands it is known that Fgf10 is expressed in the mesenchyme and signals to the epithelium (Bhushan et al., 2001; Elghazi et al., 2002; Miralles et al., 1999). In mammals, little is known about pancreatic expression and action of Fgf ligands capable of activating the “c” splice forms of the Fgf receptors. Previous work has demonstrated that Fgf9 is expressed in the epithelium of many developing organs of endodermal origin as well as in the mesothelium that surrounds the mesenchymal component of these organs (Colvin et al., 2001; Zhang et al., 2006). Fgf9 typically signals to the mesenchymal component through the “c” splice forms of FGFR1-3 during embryonic development of these organs. We previously found Fgf9 mRNA to be expressed in embryonic pancreas by RT-PCR (Dichmann et al., 2003) and in situ hybridization revealed that Fgf9 mRNA was expressed in E10.5 mesenchyme and mesothelium (Hecksher-Sorensen et al., 2004). We therefore analyzed expression levels of Fgf9 and Fgf10 mRNA in our E11.5 epithelial and mesenchymal fractions by qPCR. As expected, we found Fgf10 mRNA to be enriched in the mesenchymal fraction and similarly we found Fgf9 mRNA to be enriched in this fraction (Fig. 1C). In order to perform a similar analysis at E13.5, the time where beta cells begin to form in appreciable numbers, we FACS sorted GFP+ and GFP- cells from micro-dissected E13.5 Pdx1-GFP embryonic pancreas and isolated RNA from both fractions, which was subsequently processed for qPCR analysis. The purity of each fraction was again determined by analyzing the expression of known epithelial and mesenchymal markers (Fig. 2A). We found high levels of glucagon mRNA in the GFP+ fraction which may appear surprising. However, glucagon-producing cells arise from Pdx1+ epithelium (Herrera, 2000) and residual Pdx1 immunoreactivity can be seen in newly born glucagon-producing cells (Jensen et al., 2000). Given the long half-life of GF it is therefore not surprising to find glucagon mRNA in the GFP+ fraction. qPCR analysis of the GFP+ and GFP- fractions showed that Fgf9 and Fgf10 transcripts are enriched in the GFP+ fraction as expected for mesenchymally expressed genes (Fig. 2B), and confirms the absence of Fgf9 message in the pancreatic epithelium.

1.2. Spred1/2 and Sprouty2/4 expression depend on FGFR activity and is stimulated by Fgf9

Four mammalian Sprouty and three Spred genes have been identified in mammals. We performed standard RT-PCR to determine which Spred and Sprouty members were expressed in developing E12.5 pancreas. Transcripts for all seven genes were detected although Spry1, Spry3, and Spred3 were expressed at a very low level (Fig. 3A). qPCR experiments confirmed these expressions and indicated that Spred1 and Spry2 were five times and eight times more abundant than Spred2 and Spry4, respectively (data not shown). At the protein level, the pancreatic expression was confirmed for Spred1 and Sprouty2 and appeared to decrease as development progressed. Spred2, Sprouty3 and Sprouty4 proteins could not be detected (Fig. 3B).

In order to determine more precisely the pancreatic localization of the strongly expressed Spred and Sprouty members, we performed qPCR of isolated E11.5 epithelial and mesenchymal fractions and E13.5 Pdx1-GFP+ and -GFP- fractions. As shown in Fig. 4, Spred1 and Spry2 transcripts were enriched in the E11.5 and E13.5 mesenchymal fractions. The presence of FGFR “c”-isoforms and Fgf9 in the pancreatic mesenchyme, suggested that Fgf9 could regulate Sprouty and Spred.
expression in this compartment. We therefore tested if different Fgf's that signal either exclusively or preferentially through the "b"- or "c"-isoforms of the FGFR (Fgf7, Fgf10 or Fgf2, Fgf4, and Fgf9, respectively) could stimulate Spred and Sprouty expression in explants of embryonic pancreas. Spred1/2 and Spry2/4 expression remained constant after Fgf7 and Fgf10 treatment, with the exception of Spry4, that showed a slight decrease after FGF7 stimulation (Fig. 5A). In contrast, treatment with the "c"-isoform activators Fgf2 and Fgf9 resulted a significant increase of Spred1/2 and Spry2/4 with the highest fold stimulation detected for Spry2 and -4. Fgf4 treatment also resulted in increased Spry2/4 expression but was less potent than Fgf2 and -9.

To test if Fgf-signalling was required for pancreatic Spred1/2 and Spry2/4 expression we used two chemical inhibitors, SU5402 and U0126 which inhibit the kinase activity of FGFR and MEK1/2, respectively. We verified that treatment with these compounds inhibited phosphorylation of Erk1/2 in pancreatic explants (Fig 5B). Importantly, the basal Erk1/2 phosphorylation we detected in absence of exogenous Fgf was also reduced by both inhibitors. We then tested if the Fgf2 stimulated Spred1/2 and Spry2/4 expression was blocked by these inhibitors. Indeed, the presence of SU5402 completely blocked Fgf2 induced Spred1/2 and Spry2/4 expression in pancreatic explants, while the MEK inhibitor U0126 partially inhibited it (Fig 5C). Importantly, unstimulated expression of Spred1/2 and Spry2/4 was also reduced by both inhibitors, suggesting that endogenous Spred1/2 and Spry2/4 expression is regulated by Fgf signalling.

2. Discussion

To investigate the possible involvement of Spred and Sprouty members in murine pancreas development, we undertook a PCR screen for all known members of this family. Among them, Spry2 and Spred1 were the most highly expressed as measured by qPCR and the only ones detectable at the protein level. By two independent methods of separation, we found their expression to
expression in the pancreas. Additional input to Spred and Sprouty promoters from other RTK pathways might explain that Spred1, which we found to have the highest expression level of all the Spred and Sprouty genes in pancreatic mesenchyme, is the least responsive to treatment with Fgf9. Nevertheless, inhibition of FGFR kinase activity still attenuated Spred1 expression. Conversely, Spry4 which showed the lowest expression levels was most responsive to Fgf9 treatment. Since our expression data are based on qPCR analyses of separated epithelial and mesenchymal components we cannot accurately pinpoint the site of Fgf9 expression. Most endoderm-derived organs express Fgf9 in the epithelial (endodermal) component as well as the mesothelium surrounding the associated mesenchyme (Colvin et al., 1999) but our data reveals that the pancreas stand out among these as an organ where Fgf9 is not expressed in the epithelium. It is possible that the mesenchymal Fgf9 expression we detect is in fact solely derived from the mesothelium, and that the mesenchymal cells responding to Fgf9 in vivo are those closest to the mesothelium which may represent in minor population of all the mesenchymal cells. Thus, treatment of pancreatic explants with Fgf9 may activate signalling in mesenchymal cells that have not previously been exposed to Fgf9 in vivo. The fact that we observe a strong enrichment for Fgf9 message (~10-fold) compared to Fgf10 message (~2.5-fold) in mechanically separated epithelial and mesenchymal fractions is consistent with Fgf9 expression being restricted to the mesothelium since the mesenchyme that contaminates the epithelial fraction is likely to be that which is in close contact with the epithelium and not the outer mesothelial lining of the mesenchyme. Unfortunately, Fgf9 in situ hybridizations failed to yield reliable signals so we cannot conclusively demonstrate this notion.

The pancreas-specific lack of endodermal Fgf9 expression is notable and similar to endodermal Shh expression which is also specifically excluded from the pancreatic epithelium. Transgenic mis-expression of Shh in pancreatic epithelium results in transformation of the pancreatic mesenchyme into intestinal mesenchyme and as a consequence of this the pancreatic epithelium fails to grow and branch normally (Apelqvist et al., 1997). It may be interesting to examine the consequence of forced expression of Fgf9 in the pancreatic epithelium. In conclusion, we show here that the pancreatic mesenchyme express several FGFR "c"-isoforms as well as a number of Spred and Sprouty genes. Together our data indicate that Fgf signalling is active in the mesenchymal component of the pancreas also in mammals, and contributes to the regulation of pancreatic Spred and Sprouty expression. The Fgf signalling is likely to be mediated, at least partly, by Fgf9 which is expressed in the pancreatic mesenchyme, and most likely being restricted to the outer mesothelial lining of the mesenchyme.

Fig. 4. Mesenchymal expression of Spred and Sprouty transcripts. (A) qPCR analysis of Spred and Sprouty gene expression in E11.5 mesenchymal and epithelial fractions obtained after manual separation. Spred1 and 2, Sprouty2 and 4 are enriched in the mesenchymal fraction. (B) qPCR analysis of Spred and Sprouty gene expression in E13.5 GFP+ and GFP− cell populations in E13.5 pancreas from Pdx1-GFP embryos. Spred1 and Sprouty2 are found enriched in the GFP− cell population. Data represent mean ± SEM (n = 3); *p < 0.05, **p < 0.01.

be preferentially localized to the pancreatic mesenchyme. The same pattern of expression has been described for Spred genes in the murine lungs (Hashimoto et al., 2002), while Sprouty genes are reported to be most highly expressed in lung epithelium (Hashimoto et al., 2002; Maileux et al., 2001). Moreover, we have shown that their expressions are increased by Fgf2 and Fgf9 in pancreatic explant cultures. We show here that expression of Spred and Sprouty is under Fgf regulation in pancreatic explants, similar to what is observed in the lung, and this inductive effect of Fgf on Spred and Sprouty expression required a functional MAPK signalling pathway as shown previously in other systems (Minowada et al., 1999; Ozaki et al., 2001).

The present study extends previous work done mainly in the rat showing that the "c" splice forms of the FGFR is mainly expressed by the pancreatic mesenchyme (Elghazi et al., 2002; Cras-Mener and Scharfmann, 2002). Previous reports in transgenic mice have shown that mesenchymal mitotic activity is increased by Pdx1 promoter driven Fgf4 expression but not in embryos where the Pdx1 promoter drives Fgf10 expression (Dichmann et al., 2003; Hart et al., 2003; Norgaard et al., 2003). These studies indicate that directly or indirectly, the mesenchyme can respond to a "c"-isoform specific Fgf signal. Here we confirm this notion by showing that Fgf9 and Fgf2 both stimulate mesenchymal Spred1/2 and Sprouty2/4 expression. While Fgf2 can stimulate "b"-isoforms of the FGFR it is preferentially activating the "c"-isoforms (Zhang et al., 2006). Furthermore, the inability of Fgf7 and Fgf10, which are agonists of FGFR1b and -2b, to induce Spred1/2 or Spry2/4 expression further supports that Fgf2 and -9 induced Spred1/2 and Spry 2/4 expression is occurring via activation of the "c"-isoforms. Spred and Sprouty genes are induced by a number of receptor tyrosine kinase (RTK) pathways and we cannot rule out that RTKs different from FGFRs contribute to the regulation of Spred and Sprouty expression in the pancreas.

3. Experimental procedures

3.1. Mice

Pdx1-eGFP transgenic mice were generated by pronuclear microinjection of a 6.3 kb-long transgene, which was cloned by replacing the Cre coding region of Pdx1-Cre (Herrera, 2000) by the coding sequence of eGFP. Two different families were established from separate founder mice, displaying the same phenotype. Only one was used in these studies.

3.2. Culture of pancreatic buds

Dorsal pancreatic rudiments were dissected from embryonic day (E)11.5 NMRI mice, and cultivated in 40 μl hanging drops. Culture medium was DMEM/F12 + Glutamax medium supplemented with 1% heat-inactivated fetal calf serum (Invitrogen),
Pen/Strep (Invitrogen), and 0.04 mg/ml gentamycin (Invitrogen) and was renewed every day. Cultures were maintained at 37°C in a humidified atmosphere of 95% air–5% CO2. All the recombinant proteins were from R&D Systems, except FGF2 (Invitrogen) and were used at 100 ng/ml. Chemical inhibitors SU5402, and U0126 (both from Calbiochem) were dissolved in DMSO and used at a final concentration of 10 μM. Comparable DMSO (Sigma–Aldrich) concentration, i.e., 0.1% of the final volume was added in control conditions. Preparation of single-cell suspensions from dissected pancreata for fluorescence activated cell sorting (FACS).

Dissected pancreata from E13.5 Pdx1-eGFP embryos were incubated in a solution of 0.05% (vol/vol) trypsin–EDTA (Invitrogen) at 37°C for 5 min. The digestion was stopped by adding F10 medium containing 10% (vol/vol) FCS. The resulting suspension of single cells was washed twice with ice-cold PBS and filtered through a 70-μm nylon mesh. FACS of GFP-labelled cells was carried out using a FACS Aria (Becton Dickinson Biosciences, San Jose, CA).

3.3. RNA isolation, cDNA synthesis, RT-PCR and qPCR

RNA from dissected embryonic pancreas, from FACS purified cells, and from pancreatic explants was extracted with Invisorb Pen/Strep (Invitrogen), and 0.04 mg/ml gentamycin (Invitrogen) and was generated from RNA using MMLV Reverse Transcriptase (Invitrogen). PCR was carried out in a 25 μl reaction using ReddyMix from AbGene (Epsom, UK) on a PTC200 thermal cycler (MJ research, Waltham, MA) for 32 cycles, and resolved on a 2% agarose gel. qPCR (quantitative PCR) was performed using the standard SYBR® Green program and the dissociation curves were made on the Mx3005P machine (Stratagene). Quantified values for each gene of interest were normalized against the housekeeping gene glucose-6-phosphate dehydrogenase. In experiments where changes in gene expression were measured after stimulation or blockade of FGF signalling, the results are expressed as the relative expression compared to vehicle controls. Primers used were: Spry1: 5’-ATT TGG CCG AGA GTT GTT TG-3’ and 5’-TGA GAT ACC AGG GGC AAA TC-3’; Spry2: 5’-GGT TTT ATT CCA CCG ATT GC-3’ and 5’-CTG GGT AAG GGC ATC TCT TG-3’; Spry3: 5’-TGG AAA CAG GAA GGG AAT TG-3’ and 5’-CAT TGC AGA CAA AGC AAG GA-3’; Spry4: 5’-GCC AAC TGG AAG AGA GCA AC-3’ and 5’-CAC AGG AAT GTG GTG GAG TG-3’; Spred1: 5’-TTC ACC ACT GGA CAT ACC ATC GGT CGG GGC AAC TC-3’; Spred2: 5’-GCT CGC CAG GCA AC-3’ and 5’-CAC AGG AAT GTG GTG GAG TG-3’; Spred3: 5’-ATG CAC ACC ACC ACT GAC TGT AGC ATC TCT TG-3’; Spred4: 5’-GAC TCG GTG GGC GAC TCA AAG ACC TAT TGC TTA-3’ and 5’-ACG GAG GGC GCC GGC CAC TCA ATC-3’.

Fig. 5. Spred1/2 and Sprouty2/4 expression depends on FGFR and MEK-ERK signalling pathways. (A) qPCR analysis of Spred1, Spred2, Spry2 and Spry4 expression in E11.5 dorsal pancreatic buds cultured for 3 days in presence of vehicle (BSA), Fgf2, Fgf4, Fgf7, Fgf9 or Fgf10. Values are expressed as mean ± SEM (n = 3) after normalization to the vehicle control; *p < 0.05, **p < 0.01. (B) Phosphorylation status of ERK1/2 in explants stimulated as indicated for 2 h (C) qPCR analysis of Spred1, Spred2, Spry2 and Spry4 expression in dorsal pancreatic buds cultures with or without Fgf2 for 5 days in the presence or absence of SU5402 or U0126. Values are expressed as mean ± SEM (n = 4) after normalization to the vehicle control; *p < 0.05, **p < 0.01.
CAC TGG GAA GC-3; Glucagon: 5'-AGG TCT ACA AGG CAG AAA AA-3' and 5'-TCA TGA GTG TIG GCA ATG TT-3'; Vimentin: 5'-ATT TTG CCC TCG AAG GTG CTA AC-3' and 5'-TCA ACC AGA GGA AGT GAC TCC AG-3'; E-cadherin: 5'-GAT GTG GTG CCT GCC ACC ATG-3' and 5'-CTT GAT GTG GTG GCC CCA A-3'; Foxa2: 5'-GCT GCA GAC ACT TCC TAC TAC-3' and 5'-GGA GCA AGA GTG AGA CT-3'; Hgf: 5'-CCG ATG TGG TTA CTG TCT TCA-3' and 5'-CCG TCT GTA CCA AGG TTC-3'; Fgf11b: 5'-CTT GAC GTC GAA GCA CAT CTA-3' and 5'-AGA GCG GTC AAC CAT GAA G-3'; Fgf2b: 5'-CCC ATC TTC CAA CAA GGC CGT-3' and 5'-CAG AGC CAG CAC TTC TTC GTG A-3'; Fgf3b: 5'-CAA GGA GTG CAT ACC GCA GAC-3'; Fgf3b: 5'-GCA GGT GCT CAC ACC CTC ATG-3'; Fgf4: 5'-GTA CCC TCG GAC GCC ACC ACA TAC-3' and 5'-GCC GAA GGT CCT GGC GTT CAT G-3'; Pdk1: 5'-GAA CCC GAC GAA AAG AGG-3' and 5'-GGT CAA CAT CAC TGC GAC TTC-3'; G6DPH: 5'-ATG TTA TGC AGA ACC ACC TTC TCT TCA CG-3'.

3.4. Western blotting

Explants or embryonic pancreata were harvested in reporter lysates using the Promega, Madison, WI) in the presence of Halt phosphatase and protease inhibitor (Thermo Scientific, Waltham, MA). Lysates of CHO-K1 transfected with full-length plasmid specific sequence of Spred 1, Spred 2, Sprouty 2 or Sprouty 4 were used as positive controls. Antibodies against Spred 1, Spred 2 (both from K. Schuh, Wurzburg University, Germany, and Yoshimura, Kyushu University, Japan) Sprouty 2, Sprouty 3 (Abcam) Sprouty 4 (Santa Cruz), phospho ERK1/2 and pan ERK1/2 (both from Cell Signaling Technology) TFIIB (Santa Cruz) were used as primary antibodies at a 1:2000 dilution. HRP-conjugated antibody (from Santa Cruz) was used at a 1:10000 dilution.

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