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Spleen versus pancreas: strict control of organ interrelationship revealed by analyses of Bapx1−/− mice

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During early stages of pancreatic development, the mesenchyme that contributes to the spleen overlies the dorsal pancreatic endoderm. Here, we show that interactions between splenic mesenchyme and pancreas proceed via a highly orchestrated morphogenetic program. Disruption of morphogenesis, as occurs in the Bapx1(Nkx3.2)−/− embryo, results in transformation of these tissues into well-organized, ectopic gut-like structures. Bapx1 plays a crucial organizing role effecting position and separation of the spleen and pancreas to prevent this metaplastic transformation. Similar transformations occur in organ cultures employing wild-type pancreatic endoderm and spleen mesenchyme, revealing the developmental plasticity of the pancreas and that precise spatial and temporal control of tissue interactions are required for development of both organs.

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Epithelial–mesenchymal interactions are central to the development of the primitive gut tube endoderm and are necessary to form fully functional parts of the gastrointestinal tract. Such interactions come into play in a stepwise manner during development of the dorsal pancreas and begin with patterning signals from the mesectoderm. Later signals from the notochord repress endodermal SHF and thereby permit expression of pancreatic genes in the dorsal pancreatic anlage. Formation of the dorsal aorta will separate the notochord from the pancreatic endoderm and will in turn provide signals that induce insulin expression [Lammert et al. 2001] and references therein. Finally, splanchic mesenchyme will accumulate around the dorsal pancreatic bud and will promote growth and differentiation of the developing pancreatic epithelium [Kim and Hebrok 2001]. Studies indicate that this accumulated mesenchyme provides permissive, rather than instructive, cues [Edlund 2002]. Hence, the primary role of the mesenchymal signals at this stage is to stimulate proliferation of the different pancreatic components and not to determine developmental fate. Apart from being important for interaction with underlying epithelium, the mesenchyme surrounding the dorsal pancreas also contributes to development of the spleen during early stages of development [Fig. 1A; Kanzer and Dear 2001; Hecksher-Sørensen et al. 2004].

The mammalian homeobox gene Bapx1 (Nkx3.2) encodes a putative transcription factor that belongs to the Nkx gene family, most similar to the Drosophila homolog bap [Kim and Nirenberg 1989]. Bapx1-null mutants show visceral mesoderm defects manifesting as asplenia and impaired pyloric sphincter formation [Lettice et al. 1999; Tribioli and Lufkin 1999; Akazawa et al. 2000]. To better understand the dynamic developmental relationships between the spleen and pancreas, we have analyzed the Bapx1-null mutant, a model in which direct interaction between spleen and pancreas can be addressed. Here we show that heterotopic tissue outgrowths with the differentiated characteristics of gut form in the mutant. These are derived from the transformation of pancreatic endoderm and adjacent mesenchyme. Metaplasia was proposed as the term to cover, in vertebrates, the transformation of one developing body part to another (Slack 2004). This metaplastic transformation of embryonic pancreas to intestinal-like tissue is due to protracted interactions with spleen mesenchyme, which we show both in vivo and in vitro. In all, our data suggest that during morphogenesis, control of organ position and physical separation of tissue types, in this case spleen from pancreas, constitutes a crucial mechanism to avoid signaling that otherwise result in transformation of the tissues.

Results and Discussion

Impaired separation of spleen and pancreas in Bapx1−/− mice

Bapx1 is initially expressed in the mesenchyme flanking the dorsal and lateral aspect of the developing pancreas (Fig. 1B). Shortly thereafter, the expression shifts to a more distal leftward position, and by embryonic day 12.5 (e12.5), it is restricted to the spleen anlage [Fig. 1C; Hecksher-Sørensen et al. 2004]. Bapx1 expression could not stage be detected in the gastrointestinal or pancreatic endoderm [Fig. 1B,C]. Hence, the Bapx1 expression pattern correlates well with the described asplenic phenotype [Lettice et al. 1999; Tribioli and Lufkin 1999; Akazawa et al. 2000]. However, the fate of the splenic mesenchyme in Bapx1−/− mice has not been fully addressed.

The spleen is typically derived to form as a mesenchymal condensation within the dorsal mesogastrium adjacent to the stomach and dorsal pancreas [Patterson et al. 2000]. We examined the spatial relationship of the spleen and pancreas during development in wild-type and Bapx1−/− embryos using optical projection tomography (OPT) [Sharpe et al. 2002] to establish three-dimen-
condensation/separation of the distal mesenchyme. The splenic mesenchyme does not separate from the pancreatic endoderm (Hecksher-Sorensen et al. 2004); however, shortly after formation, the endodermal component of the cysts separate from the bulk of the pancreatic endoderm and appear as isolated structures throughout development (see Fig. 2).

To determine the nature of the cysts, we analyzed a number of markers indicative of the tissue of origin. Rudimentary splenic functions could not be accounted for,

Figure 2. Cystic structures are formed from the pancreatic endoderm in Bapx-/- mice. In all figures the cyst lumen is marked by an asterisk. [A–C] The splenopancreatic region, including stomach, from e14.5 Bapx-/- [A] and Bapx+/+ [B] embryos forming [B] and not forming [C] cystic structures. The approximate position of rudimentary splenic mesenchyme is marked by broken line [C]. [D] Whole mount of pancreatic cysts labeled for HNF3β (red) and smooth muscle α-actin [ASMA, green]. [E] OPT section of specimen seen in D showing continuation between a developing cyst and the pancreatic endoderm (*). The cyst marked ** has pinched off from the endoderm. Concomitant with cyst formation, smooth muscle is induced in the splenic mesenchyme (arrowhead). [F] Iso-surface reconstruction based on HNF3β signal of OPT sections as seen in E. Blue plane corresponds to E. [G] Cryosection through two cysts labeled for E-cadherin (red) and Ptf1A (green). Ptf1A is expressed in the pancreatic endoderm but not in the cysts. [H] Photomicrograph of the splenopancreatic region and stomach from e18.5 Bapx-/- and Bapx+/+ embryos. [i–K] Cryosections through the cyst of the center mutant seen in H labeled for DAPI (blue, I) and ASMA (green, J) and C-kit (red, K). K corresponds to white box in I and J and shows overlay of the ASMA and C-kit signal. C-kit-positive cells are intercalated in the muscle layer surrounding the cyst (arrowheads, K). dp indicates dorsal pancreas; vp, ventral pancreas; spl, spleen; st, stomach; and d, duodenum. Bar, 50 µm (G), 108 µm (I, J), 23 µm (K).
and markers for endothelium (Flk1) and infiltrating red blood cells (CD45) revealed no resemblance to the highly vascularized and blood cell infiltrated spleen (data not shown). Moreover, at both stages analyzed (e14.5 and e18.5) the endodermal components of the cysts were negative for a battery of pancreatic antibody markers including carboxypeptidase A, amylase, insulin, glucagon, somatostatin, Isl1, Ngn3, PDX1 (Ipf1), HB9, and Ptf1a (p48) [Fig. 2G; data not shown]. In contrast to pancreatic mesenchyme, intestinal and stomach mesenchyme becomes organized into a bilayer of smooth muscle cells. This is intercalated by c-kit-expressing interstitial cells of Cajal (ICC), which are believed to mediate peristaltic movements [Huizinga et al. 1995; Bernex et al. 1996]. Interestingly, formation of a smooth muscle bilayer was detected adjacent to the developing cysts in Bapx1−/− mice (Fig. 2D,E,I). Similar to gut smooth muscle, this tissue became intercalated by c-kit-positive cells, indicating the presence of ICCs (Fig. 2I–K). Together these data show that in Bapx1−/− mice, ectopic cysts differentiate from pancreatic epithelial cells, which lose the molecular and morphological features of pancreatic endoderm. Subsequently, the adjacent mesenchyme organizes itself around the transformed epithelium and displays characteristics of differentiated gut mesenchyme.

The Shh signaling pathway is activated in Bapx1−/− dorsal pancreas

SHH and IHH, members of the hedgehog family of signaling molecules, are expressed in the embryonic gut endoderm and are key mediators in the development of both the gut endoderm and mesoderm [Ramalho-Santos et al. 2000]. Low levels of Ihh expression have been reported in the developing pancreatic anlagen, whereas Shh is excluded [Ahlgren et al. 1996; Apelqvist et al. 1997; Hebrok et al. 2000]. Moreover, ectopic expression of Shh in the pancreatic endoderm generates a mixed pancreatic-intestinal phenotype [Apelqvist et al. 1997]. To explore the possibility that hedgehog signaling contributes to the gut-like phenotype, we analyzed for expression of Shh, Ihh, and the putative hedgehog receptor Ptc in cysts from e14.5 embryos. Expression of these could not be detected in the wild-type pancreatic epithelium/mesenchyme or in the splenic primordium by in situ hybridizations (Fig. 3A–C). However, expression of Shh, but not Ihh, was detected in the epithelial component of the transformed pancreatic epithelium in Bapx1−/− mice (Fig. 3E,F). In addition, the surrounding mesenchyme expressed Ptc, indicative of active hedgehog signaling [Fig. 3G]. These results provide evidence that the SHH signaling pathway is ectopically activated in cells of the dorsal pancreatic epithelium of Bapx1−/− mice. Moreover, pancreatic epithelial transformation, including altered cell arrangement and gene expression, in turn results in gut specific differentiation of surrounding mesenchyme.

The timing of ectopic SHH expression influences cyst formation

In mutant embryos that did not form cystic structures, the abundant mass of unorganized splenic mesenchyme described previously could not be observed after e15.5–e16 [Fig. 2H; data not shown.] To determine the fate of this tissue, we analyzed for apoptosis by using the TUNEL assay, which revealed a high degree of apoptotic cells in the Bapx1-deficient splenic mesenchyme at e14. At the same stage, mutant embryos, which produced cysts, did not exhibit abnormal apoptosis in the spleno-pancreatic region [Supplemental Fig. S1]. SHH induction of gut-specific differentiation may thus rescue the splenic mesenchyme from cell death in the Bapx1-null mutants. We suggest that timing is crucial to the transformation of the pancreatic epithelium and involves a fine balance between cyst formation and splenic mesenchymal cell death. Hence the penetrance of the phenotype is dependent on the sequence of events, i.e., epithelial SHH expression must precede mesenchymal cell death to produce detectable ectopic cysts.

Splenic mesenchyme mediates transformation of pancreatic epithelium

In Bapx1-null mice, cyst formation commences at a stage [approximately e140] when the amount of pancreatic mesenchyme, relative to pancreatic epithelium, is reduced both during normal development and in Bapx1 mutants [Jensen 2004]. Hence, signaling from the tightly associated rudimentary splenic mesenchyme is the principal mediator of the Bapx1 mutant phenotype. To elucidate the capacity of Bapx1−/− splenic mesenchyme (bSM) to cause pancreatic epithelial transformation and to more definitively prove lineage of the cyst epithelial component, a set of in vitro culture experiments was executed. Splenopancreatic tissue was analyzed from e13.5 embryos, which is approximately half a day before pancreatic transformations first commence in vivo. Similar to previous reports [Ahlgren et al. 1996; van Eyll et al. 2004], wild-type whole pancreatic explants as well as wild-type pancreatic epithelium (WPE) recombined with stage-matched wild-type pancreatic mesenchyme (WPM) mimicked normal in vivo development. Thus, branching morphogenesis as well as endocrine and exocrine differentiation could be accounted for in both types of culture [Fig. 4A,H–L, n = 16; data not shown]. Similarly, explants of Bapx1−/−-derived pancreatic epithelium (bPE) and WPM developed apparently normal [Fig. 4B,M–Q, n = 6].
In contrast, recombining wPE with bSM, led to formation of cystic structures in 58% of the explants [Fig. 4C, n = 19]. A subset of these explants developed well-defined cysts and started to exhibit peristaltic movements toward the end of the culture period [Fig. 4C, Supplemental Movie 2]. Similarly, explants of isolated cysts derived from Bapx1−/− embryos at e14.5 displayed peristaltic contractions [Fig. 4D, Supplemental Movie 3]. Cystic structures were never observed in explants composed of wPE+wPM, bPE+wPM, or whole wild-type pancreas [Fig. 4A,B, data not shown]. Analysis for Shh expression by RT-PCR validated the intestinal character of the transformed explants [Fig. 4E; data not shown]. Notably, Bapx1−/− cyst explants also expressed intestinal fatty acid binding protein [iFABP] [data not shown], a marker for intestinal differentiation [Gordon et al. 1985]. Similar to the cysts formed in vivo in Bapx1−/− mice, immunohistochemical analyses consistently failed to detect pancreatic markers such as Pdx1, Ptf1a, insulin, glucagon, and amylase in the cystic epithelium of the wPE+bSM explants [data not shown]. Hence, these data provide evidence that splenic mesenchyme of Bapx1−/− origin holds the capacity to transform intestinal-like differentiation of wild-type pancreatic epithelium.

Wild-type spleen mesenchyme induces cyst formation

Exogenous activin A disrupts lobulation of the developing pancreas in culture and vesicles are pinched off at high concentrations [Ritvos et al. 1995]. Moreover, a recent report showed that in vitro exposure of pancreatic buds in culture to activin A, but not activin B, develops intestinal characteristics in a dose-dependent manner [van Eyll et al. 2004]. The observed differentiation of intestinal tissue from pancreas by supplemented activin A closely resembles our observations including onset of Shh and iFABP expression. Activin A is constitutively expressed by the mesenchymal spleen stroma [Shoham et al. 2003]. bSM and wild-type splenic mesenchyme [wSM] isolated at e13.5 were positive for the Activin βA subunit by RT-PCR (Fig. 4G). Hence, these findings raise the possibility that short-range Activin A signaling by Bapx1−/− spleen mesenchyme may mediate differentiation of intestinal tissue from pancreatic endoderm in vivo and in vitro. Regardless of the signaling mechanisms, disrupted spleen morphogenesis in the Bapx1−/− mice leads to the abnormal distribution of the splenic mesenchyme in short range of the pancreatic epithelium invoking endodermal transformation. To examine the significance of spleen morphogenesis on pancreatic development, we recombined wPE with wSM. This combination resulted in formation of cystic structures (60.5%) [Fig. 4E, n = 38]. The wPE+wSM combination, similar to the epithelium of the cystic structures formed in the wPE+bSM combination, did not express markers for pancreatic progenitors or differentiated pancreatic cell types [Fig. 4R–V]. Hence wild-type splenic mesenchyme has a similar capacity to effect transformation of pancreatic epithelium.

Ptf1a is normally expressed in progenitors of pancreatic endocrine, exocrine, and duct cells but not in the duodenum [Kawaguchi et al. 2002] and appears to be an important step in lineage determination in favor of a pancreatic as opposed to a duodenal fate [Kawaguchi et al. 2002]. Neither in the transformed pancreatic epithelium, derived from Bapx1−/− embryos, nor in the cystic structures formed in the in vitro recombination experiments could we identify the expression of pancreatic markers including Ptf1a [see Figs. 2G, 4S–V]. Thus, down-regulation or blocked expression of Ptf1a in pancreatic progenitor cells may be a prerequisite for these to convert to a more general gut endodermal fate. Notably, in some lower vertebrates, as in Ptf1a-deficient mice [which lack exocrine parenchyma], pancreatic endocrine cells colonize the spleen [Krapp et al. 1998]. Hence, it is likely that cyst formation, due to interaction with splenic mesenchyme as observed here, does not involve cells committed to an endocrine lineage.

Direct spleen–pancreas interaction appears unique for the Bapx1−/− mouse model

Several mouse models exhibiting varying degrees of disturbed spleen development have been described. Analyses of mice deficient for Hox11 [Dear et al. 1995; Kanzler and Dear 2001], Pbx1 [Lu et al. 2000], Pod1 [Lu et al. 2000], WTI [Herzer et al. 1999], and Nkx2.3 [Pabst et al. 1999] have shown that these genes, although not required for initial spleen specification, are essential for the expansion and/or survival of spleen precursors. These mutants have not been reported to show pancreatic abnormalities. In Hox11−/−, Pbx1−/−, and WTI-null mutant mice, spleen organogenesis commences more or less normally up to approximately e13. This includes condensation and separation of splenic mesenchyme and
pancreatic epithelium [Herzer et al. 1999]. In Pod1 mutants, splenic precursors are specified but do not expand to give rise to a recognizable organ. In contrast to Bapx1-null mice, the region of the developing spleen in Pod1 mutants was virtually devoid of cells by e13.5 [Lu et al. 2000]. Finally, in Nkx2.3-deficient mice, the splenic phenotype is primarily characterized by migration and homing defects of lymphocytes [Pabst et al. 1999]. Hence, none of the above mutants appear to present splenic mesenchyme in close contact to the dorsal pancreatic epithelial primordium at the time (i.e., e14–e14.5) of pancreatic epithelial transformation in the Bapx1 mutant.

A model for Bapx1 in controlling splenopancreatic interrelationship

The data presented here show that the metaplastic conversion of organ type requires the participation of two normally unrelated tissue types, i.e., pancreatic endoderm and splenic mesenchyme, to generate the heterotopic intestinal-structures. In Bapx1-null mice, pancreatic epithelial cells undergo morphological and molecular changes, including onset of markers for gut specific differentiation, whereas splenic mesenchyme transforms to constitute the adjacent smooth muscle. We show that during normal development, Bapx1-dependent morphogenetic movement and tissue separation of pancreas-associated mesenchyme is key to facilitate spleen and pancreas formation (summarized in Fig. 5). The separation of spleen and pancreas prevents aberrant signaling otherwise affecting development of these organs. Transformation of pancreatic epithelium was induced by provision of either wild-type or Bapx1−/−, splenic mesenchyme, both of which express Activin A, a good candidate for the signaling molecule involved. The specific targets for Bapx1 in the splenic mesenchyme are yet to be identified, and misregulation of other signaling molecules may well contribute to the phenotype. Seemingly, the pancreatic epithelium possesses a high degree of cellular plasticity and may be respecified by external stimuli as provided by splenic mesenchyme regardless of source. Furthermore, cyst formation may be induced in a dose-dependent manner, a parameter difficult to control in an explant recombination system, which could further influence the degree of cyst formation in vitro. Albeit a larger region of the distal pancreas in Bapx1−/− mice is surrounded by splenic mesenchyme, the cystic structures formed usually had no more than one to two foci. A possible explanation is that induction of gut-specific differentiation in this mesenchyme could serve as a negative feedback mechanism, preventing additional interactions.

Notably, pancreatic transformation with associated expression of SHH, in many respects, resembles diverse malignancies in this region. Thayer et al. (2003) showed that Shh expression in pancreatic adenocarcinomas and its precursor lesions, suggesting that SHH signaling plays an important role in the genesis of this cancer type. In fact, cases of pathologic spleen ruptures, caused by direct invasion of pancreatic tail (i.e., dorsal) adenocarcinomas, have been described [Smith et al. 2004]. Moreover, reports of epithelial splenic cysts, often located in accessory spleens of the pancreas [Choi et al. 2000; Horibe et al. 2001], and gastric duplication cysts located in the splenopancreatic region [D’Journo et al. 2004] show striking similarities with the cysts that occur in Bapx1 mutant embryos.

Material and methods

Animals and isolation of embryos
Bapx1−/+; Bapx1−/−, and Bapx1−/− mice were obtained from our local breeding colony of Bapx1−/− mice (Lettice et al. 1999). For isolation of embryos, the morning of the vaginal plug was designated e0.5. Wild-type tissue used in the explant study was isolated from C57Bl/6 × CBA crosses. C57Bl/6 females and CBA males were purchased from Bomholtgaard.

Embryonic explants
For details, see Supplemental Material.

PCR detection
Total RNA was purified by using NucleoSpin RNA II kit (BD Bioscience), and cDNA was synthesized by using Transcriptor First Strand cDNA Synthesis kit (Roche). Shh, Activin βA, and iBAP were detected using Taq DNA polymerase (Roche). The primers used for Shh were 5′-ATG CTTGGCTGGCCTGGCTGTGGAA-3′ and 5′-TGCTGTCACAGCGACT TCCTCA-3′ (241 bp); for Activin βA, 5′-GATCGAAGAACTCTTAC GTTG-3′ and 5′-TACGGAAAGCCACACTCTCTG-3′ (197 bp); for iBAP, 5′-CGGCACGTGGAAAGTACGC-3′ and 5′-CGGCTGCAGCTGTA GGAGG-3′ (197 bp); and for GAPDH, 5′-ACGGCAAATTCCAC GCCACAG-3′ and 5′-GTCAGCTGACCCCTCCTCAATG-3′ (371 bp).

Immunohistochemistry and in situ hybridizations
For details, see Supplemental Material.

Optical projection tomography analysis
Optical projection tomography was carried out as described [Sharpe et al. 2002].

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