The splanchnic mesodermal plate directs spleen and pancreatic laterality, and is regulated by *Bapx1/Nkx3.2*

Jacob Hecksher-Sørensen[1,*,†], Robert P. Watson[1,*], Laura A. Lettice[1], Palle Serup[2], Lorraine Eley[3], Carlo De Angelis[1], Ulf Ahlgren[1,‡] and Robert E. Hill[1,§]

[1]Comparative and Developmental Genetics Section, MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK
[2]Department of Developmental Biology, Hagedorn Research Institute, Niels Steensens Vej 6, 2820 Gentofte, Denmark
[3]The Institute of Human Genetics, The International Centre for Life, Central Parkway, Newcastle upon Tyne NE1 3BZ, UK
*These authors contributed equally to this work
†Present address: Department of Developmental Biology, Hagedorn Research Institute, Niels Steensens Vej 6, 2820 Gentofte, Denmark
‡Present address: Umeå Centre for Molecular Medicine, Umeå University, 901 87, Umeå, Sweden
§Author for correspondence (e-mail: bob.hill@hgu.mrc.ac.uk)

Summary

The mechanism by which left-right (LR) information is interpreted by organ primordia during asymmetric morphogenesis is largely unknown. We show that spleen and pancreatic laterality is dependent on a specialised, columnar mesodermal-derived cell layer referred to here as the splanchnic mesodermal plate (SMP). At early embryonic stages, the SMP is bilateral, surrounding the midline-located stomach and dorsal pancreatic bud. Under control of the LR asymmetry pathway, the left SMP is maintained and grows laterally. Mice carrying the dominant hemimelia (*Dh*) mutation lack the SMP. Significantly, the mice are asplenic and the pancreas remains positioned along the embryonic midline. In the absence of *Fgf10* expression, the spleno-pancreatic mesenchyme and surrounding SMP grow laterally but contain no endodermal component, showing that leftward growth is autonomous and independent of endoderm. In the *Bapx1⁻/⁻* mutants, the SMP is defective. Normally, the SMP is a source for both Fgf9 and Fgf10; however, in the *Bapx1* mutant, *Fgf10* expression is downregulated and the dorsal pancreas remains at the midline. We conclude that the SMP is an organiser responsible for the leftward growth of the spleno-pancreatic region and that *Bapx1* regulates SMP functions required for pancreatic laterality.

Key words: LR asymmetry, *Bapx1*, Pancreas, Spleen, SMP, Fgf regulation, Mouse

Introduction

From the initial bilaterally symmetrical mammalian embryo, left-right (LR) asymmetries appear throughout affecting the structure and location of internal organs (Capdevila et al., 2000; Hamada et al., 2002; Bisgrove et al., 2003). Such visceral asymmetries exist in all vertebrates, suggesting that many of the mechanisms that generate sidedness are conserved during evolution (Boorman and Shimeld, 2002). The mechanism that establishes LR positional information has been intensively investigated. LR positional information initiates near the embryonic organiser by a process of nodal flow and is subsequently relayed to the lateral plate mesoderm (LPM) (Nonaka et al., 1998; Capdevila and Belmonte, 2000; Nonaka et al., 2002). Several genes have been identified which are necessary for establishing LR asymmetries many of which show remarkable side-specific expression patterns in the LPM. However, one question that has received little attention is how this information is ultimately conveyed to the organ primordia, resulting in the implementation of programs of asymmetric organ morphogenesis (Capdevila and Belmonte, 2000).

The normal disposition of organs is called situs solitus.

Disruptions of organ situs are common and heterotaxia conditions cover a broad range of gastrointestinal and cardiac defects (Burn, 1991; Aylsworth, 2001). In situs inversus complete mirror-image reversal of situs occurs. More commonly individuals have partial situs malformations owing to randomisation of patterning information and only a few organs are affected (situs ambiguus or heterotaxy). The mesenchymally derived spleen is normally situated on the left side of the abdominal cavity and as such is a readily identifiable landmark for detecting extensive laterality defects (Aylsworth, 2001). Conditions such as asplenia, and polysplenia syndromes are often associated with situs ambiguous, indicating an underlying, fundamental defect of asymmetric organogenesis (Bowers et al., 1996). Loss of splenic tissue (asplenia or rudimentary spleen) relates to bilateral right sidedness called right isomerism, whereas the contrasting phenotype of additional splenic tissue (polysplenia, either as additional splenules or multilobulated single spleen) relates to bilateral left sidedness (left isomerism). Hence, spleen abnormalities are frequently associated with situs malformations of other organ systems dependent on LR signalling (Ivermark's syndrome) (Ivemark, 1955).

In this report, we have examined two asplenic mouse mutations, dominant hemimelia (*Dh*) and the *Bapx1*-targeted disruption, to investigate the relationship of spleen development and LR asymmetry. *Dh* is an established, spontaneously derived mouse mutation that disrupts visceral and limb development (Green, 1967). The *Bapx1* gene (also referred to as *Nkx3.2*) is a member of the NK family of homeobox-containing genes (Tribioli et al., 1997) first described in *Drosophila* and is most closely related to the *Drosophila* bagpipe (*bap*) gene (Azpiazu and Frasch, 1993). Targeted mutations of *Bapx1* results in loss of the spleen and vertebral defects (Lettice et al., 1999; Tribioli and Lufkin, 1999; Akazawa et al., 2000). Examination of visceral development in *Dh* and *Bapx1* mutants led to the identification of a mechanism whereby the primordial spleno-pancreatic mesoderm that surrounds the gut endoderm controls localised asymmetric growth.

Materials and methods

Embryos and immunohistochemistry

Postimplantation mouse embryos were collected at the desired stages, considering the day of the vaginal plug as E0.5, and were genotyped as described (Lettice et al., 1999). Whole-mount immunohistochemistry was carried out as described (Sharpe et al., 2002), while combined immunohistochemistry and in situ hybridisation was performed as detailed by Heckscher-Sørensen and Sharpe (Heckscher-Sørensen and Sharpe, 2001). Following staining, tissue was embedded, sectioned and counterstained as described by Heckscher-Sørensen and Sharpe (Heckscher-Sørensen and Sharpe, 2001). Alternatively, samples were embedded in 1% LMP agarose and analysed by Optical Projection Tomography as described (Sharpe et al., 2002). The PDX1 and ISL1 antibodies have been described previously (Ahlgren et al., 1997).

Probes and in situ hybridisation

DIG-in situ hybridisation was performed essentially as described by Wilkinson (Wilkinson, 1992). Prior to staining, the embryos were washed twice in 0.1 M Tris (pH 8.2) for 30 minutes and the signal was visualized in 2 ml 0.1 M Tris (pH 8.2) containing one Fast Red tablet (Roche). The probes for *Bapx1*, *Nkx2.5*, *Hox11* (*Tlx1* – Mouse Genome Informatics), *Wt1*, capsulin (*Tcf2l* – Mouse Genome Informatics), *Barx1*, *Pitx2*, *Fgf9*, *Fgf10* and *Fgfr3* have been described previously (Mundlos et al., 1993; Peters et al., 1993; Roberts et al., 1994; Tissier-Seta et al., 1995; Semina et al., 1996; Bellusci et al., 1997; Lu et al., 1998; Colvin et al., 1999; Lettice et al., 2001).

BrdU labelling

E10.5 embryos were labelled with BrdU by intraperitoneal injection of 200 µl BrdU (10 mg/ml) into pregnant females. After a 30 minute labelling period, the embryos were dissected out and fixed in 4% PFA. Embryos were then embedded in paraffin wax and sectioned. After rehydration, sections were trypsinised for 10 minutes at 37°C, washed in PBS and then incubated for 10 minutes in 1 N HCl. Incorporation of BrdU was detected using anti-BrdU monoclonal antibodies and Alexa Fluor® 488 goat anti-mouse secondary antibody (1:200 dilution; Mol. Probes). Finally, sections were counterstained with Propidium Iodide (1:5000).

Results

Initiation of splenogenesis and the relationship to the stomach and pancreas

The spleen is a wholly left-sided organ in the wild-type mouse, closely associated with the pancreas, and located on the left lateral side of the stomach. Here, we examine the location of the pre-splenic rudiment relative to the stomach and pancreatic primordia at the initiation of organogenesis. Histological analysis of late gestation mammalian embryos indicates that the splenic rudiment is recognisable as a single condensation of mesenchyme along the left side of the mesogastrium dorsal to the stomach (Thiel and Downey, 1921). Analysis of genes expressed in the mouse spleen, such as *Hox11* (Roberts et al., 1994; Dear et al., 1995), enable the tracing of spleen development to earlier embryonic stages. By E11.5, *Hox11* expression is established as left-handed and in the dorsal mesogastrium lateral to the stomach (Kanzler and Dear, 2001).

We have identified the initial lateral position of the pre-spleen rudiment at a stage before the spleen is associated with the stomach. At E10.5, cross-sections taken through the embryo in the region of the developing forelimb reveal the spatial relationship between the stomach (Fig. 1A,B) and dorsal pancreas (Fig. 1A,C) during the early stages of leftward growth. At this stage, *Hox11* expression is detected in the dorsal mesenchyme positioned on the left-lateral side, but lying posterior to the stomach primordium (Fig. 1D). The pancreas-specific anti-PDX1 antibody shows that this mesenchyme also supports the dorsal pancreatic bud (Fig. 1D) lying ventral to the splenic rudiment.

Expression of other genes implicated in spleen development confirmed that this region of the embryo participates in spleen formation. In *Xenopus*, *Nkx2.5* is expressed during early stages of spleen development (Patterson et al., 2000). In mouse embryos, *Nkx2.5* expression was detected at E10.5 in two distinct domains around the dorsal pancreatic bud; a ventral domain and a dorsal domain that overlaps *Hox11* (Fig. 1E). Targeted mutations in capsulin/*Pod1* (Lu et al., 2000) and *Wt1* (Herzer et al., 1999) show that both genes are required in spleen formation. The expression patterns of *Wt1* and *capsulin* overlap with those of *Hox11* and *Nkx2.5* in the dorsal region of the mesenchyme (Fig. 1F,G), indicating that this region will ultimately give rise to the spleen. By E11.5, the splenic rudiment, which continues to express *Wt1* (Herzer et al., 1999), capsulin (Lu et al., 2000), *Nkx2.5* and *Hox11* (Dear et al., 1995; Kanzler and Dear, 2001; Roberts et al., 1994) (data not shown), is located in the dorsal mesogastrium, which is now lateral to the stomach. While subsequent splenic development occurs in close association with the morphogenesis of the stomach, we show that the pre-spleen mesenchyme and endodermal-derived dorsal pancreatic bud are closely associated posterior to the stomach at an early developmental stage.

A distinct columnar epithelium surrounds the spleno-pancreatic mesenchyme

An outer layer of epithelium surrounding the splenopancreatic mesenchyme was noted in the E10.5 embryos (broken lines in Fig. 1D,E). Earlier stages were examined to establish when this tissue originates. The splanchnic mesoderm is the region of the lateral plate mesoderm (LPM) that surrounds the gut. In the region of the dorsal pancreatic bud, we found that fluorescently tagged phalloidin detects the accumulation of f-actin at the boundary between the endoderm and splanchnic mesoderm and at the outer margin of the splanchnic mesoderm, thus outlining a thickened epithelial structure (Fig. 2A,B). The splanchnic mesoderm surrounds the foregut and appears as a bilateral, highly organised layer of elongated cells ~50-60 µm
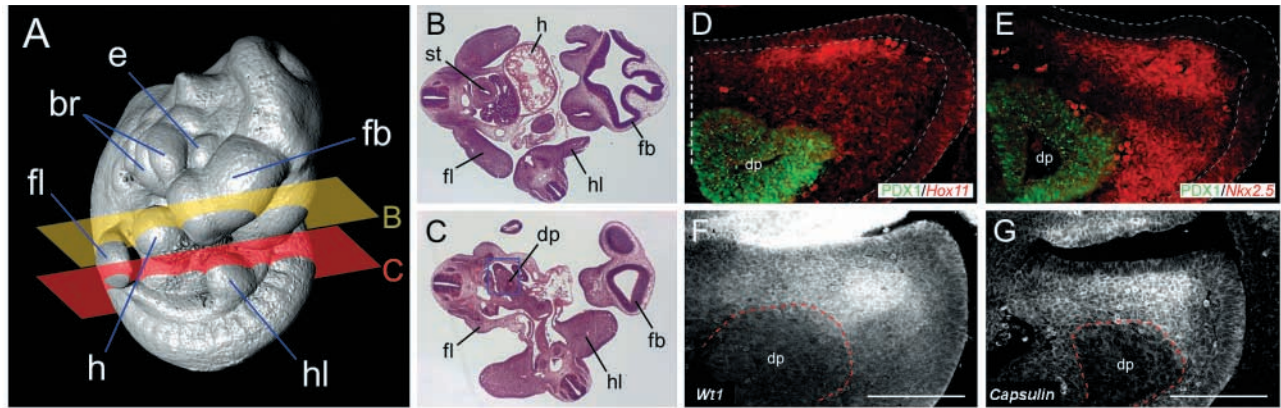
Fig. 1. Advent of the spleno-pancreatic mesenchyme and the relationship to the SMP. (A) Reconstruction of an E10.5 embryo following analysis by optical projection tomography (OPT). Yellow and red boxes indicate the planes of the sections shown in B and C. (B,C) Cross-sections through an E10.5 embryo in the region of the stomach (B) and dorsal pancreas (C). Boxed region in C indicates the embryonic position of the images shown in D-G, and of comparable images presented throughout the manuscript. (D-G) Expression of early spleen marker genes in transverse sections through the dorsal pancreatic bud in E10.5 embryos. In situ hybridisation showing expression of *Hox11* (D) and *Nkx2.5* (E) (seen as red signal) in combination with pancreatic marker anti-PDX1 antibody (green). The double label highlights the relationship of spleen and pancreas location in the early mesenchyme. Expression of *Wt1* (F) and capsulin/*Pod1* (G) (single label shown as white signal) overlaps *Hox11* expression domain and confirms that the dorsal mesenchyme is the region of the spleen rudiment. There is close association with the thickened mesodermal layer that does not express the spleen markers at a high level. The approximate position of the embryonic midline is indicated by the broken line in D. The thickened epithelium that surrounds the mesenchyme is outline by the broken lines in D and E. br, branchial arches; dp, dorsal pancreatic bud; e, eye; fb, forebrain; fl, forelimb; h, heart; hl, hindlimb; st, stomach. Scale bars: 150 μm.

thick that is easily distinguishable from the unorganised mesenchymal cells that compose a large proportion of the embryo. The position of the dorsal pancreatic bud was detected with the PDX1 antibody (Fig. 2A,C,D,O,P). The pancreatic bud is situated at the embryonic midline (Fig. 2A) and extends dorsally flanked tightly on both sides by the splanchnic mesoderm.

We have analysed development of this region in the asplenic dominant hemimelia (*Dh*) mouse mutant. The *Dh/Dh* mutant was of particular interest based on the original description by Green (Green, 1967), which suggested that the structural integrity of the splanchnic mesoderm was disrupted. At E9.5 in *Dh/Dh* mutant embryos, the organised splanchnic mesoderm is not apparent but is replaced by unorganised mesenchyme (Fig. 2D,E). The *Dh/Dh* mutant embryos highlight the distinct structure of the wild-type mesoderm, which, similar to the term used by Green (1967), we propose to name the splanchnic mesodermal plate (SMP).

By E10 the spleno-pancreatic region in wild-type embryos acquires a characteristic triangular shape in cross-section (Fig. 2C). The SMP is prominent and located on the left side (white arrowheads in Fig. 2C). The mesenchyme on the right side of the gut tube remains positioned near the embryonic midline and the thick epithelial plate-like structure is lost. TUNEL staining for apoptotic nuclei reveals negligible cell death in this region between the stages E9.5 and E10.5 (data not shown). This argues against apoptosis as the driving force behind the observed loss of the right-sided SMP. Cells of the right SMP persist after E9.5, most probably contributing to the underlying mesenchyme and the thin layer of cuboidal cells (indistinguishable from the mesothelial layer that lines the mesenchyme in the remainder of the coelomic cavity and shown by yellow arrowheads in Fig. 2C) that is found in place of the right SMP from E10.0 onwards. Analysis of nuclear

morphology in the spleno-pancreatic region between E9.5 and E10.5 reveals that the elongated nuclear structure that characterises the left SMP is progressively lost from the right-hand side during this period (Fig. 2F-N). By E10.0, nuclei positioned ventrally along the right margin resemble those present in the underlying mesenchyme or in adjacent mesothelia (Fig. 2I,K). At this stage, nuclei positioned dorsally along the right margin retain their elongated morphology (Fig. 2I,J). By E10.5, nuclei along the length of the right-hand margin are overtly indistinguishable from those in the underlying mesenchyme or in adjacent mesothelia (Fig. 2L-N), suggesting that the observed loss of the right-hand SMP is actually the result of a spatiotemporal change in cell morphology.

Dorsal pancreatic bud growth accompanies the asymmetric expansion of the mesenchyme such that it becomes displaced to the left of the embryonic midline. The SMP, which by bright-field microscopy appears as a translucent cell layer (indicated by broken lines in Fig. 6A), entirely encompasses the left, lateral spleno-pancreatic mesenchyme extending from the posterior half of the stomach to the pancreatic buds. During the subsequent day of development, the shape and position of the region changes dramatically and by E11.5 the thick epithelial structure is no longer detected (data not shown).

Thus, the SMP, a derivative of lateral plate mesoderm, is bilateral at initial stages. This columnar epithelium lines the gut endoderm with few intervening mesenchymal cells. Within ~12 hours the SMP is preferentially maintained on the left-hand side accompanied by lateral growth and appearance of underlying mesenchymal cells. The gut tube remains at the embryonic midline, while the dorsal pancreatic bud moves leftward accompanying the outgrowth of the SMP. Expression of *Hox11*, *Nkx2.5* and capsulin/*Pod1* in E10.5 embryos is located in the mesenchyme directly underlying the SMP, which
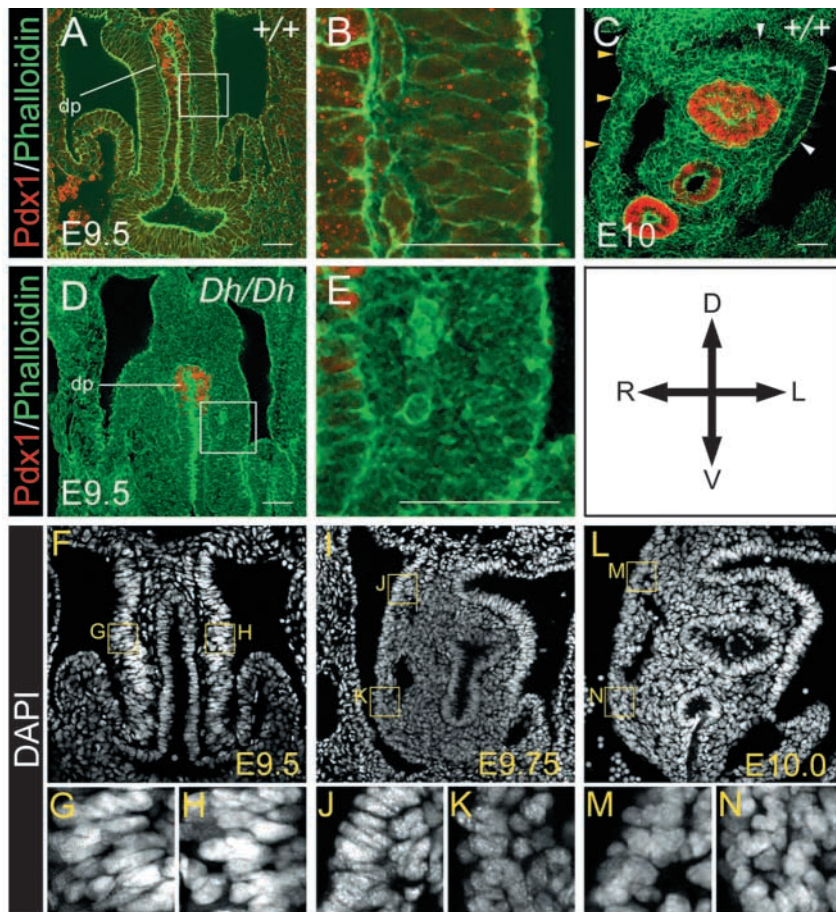
Fig. 2. SMP formation in the developing mouse embryo. (A-C) Timecourse showing the development of the SMP during mid-gestation. (A) Transverse section through an E9.5 wild-type embryo stained with antibodies for phalloidin (green) and PDX1 (red). The position of the dorsal pancreatic bud is shown with the PDX1 antibody. The white box indicates the region that is magnified (4×) in B, which highlights the organised cellular structure of the SMP that is revealed following staining for f-actin. The cells are elongated and situated perpendicular to the dorsoventral embryonic axis. (C) At E10, the SMP (white arrowheads) and spleno-pancreatic mesenchyme have grown laterally to the left. The SMP that was situated on the right side is now absent and is replaced by a thin mesothelial layer (yellow arrowheads). (D) *Dh/Dh* mutant embryos at E9.5 differ from wild type in that an unorganised mesenchymal layer of cells surround the midline-positioned dorsal pancreatic bud. The white box indicates the region magnified (4×) in E; the cells are round and densely packed. (F-N) At E9.5, the initially bilateral SMP is characterised by elongated nuclei (F-H). These are lost on the right-hand side in a ventral-to-dorsal direction over the course of 24 hours, and in their place nuclei resembling those in the underlying mesenchyme and adjacent mesothelia are observed (I-N). The yellow boxes in F,I and L indicate the regions magnified (4×) in G,H,J,K,M,N. Dorsal and ventral (in C only) pancreatic buds are shown in red (PDX1 antibody stain). dp, dorsal pancreatic bud. Scale bars: 60 µm in A,C-E.

itself does not express these genes at a high level (Fig. 1D,E,G), suggesting that the SMP is distinct from, but closely associated with, the developing spleen.

The SMP is under control of the LR signalling cascade

To determine whether SMP asymmetry is under control of the LR signalling pathway, we analysed expression of *Pitx2*, a gene known to be expressed in the left LPM during early embryogenesis (Logan et al., 1998; Piedra et al., 1998; Shiratori et al., 2001). *Pitx2* has a role in lung isomerism, heart development and rotation of the duodenum (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Liu et al., 2001). Expression of the *Pitx2* gene persists later than many other genes in the LR signalling cascade, with expression still detectable at E9.5. At this stage, before asymmetric organogenesis is apparent, *Pitx2* is expressed predominantly in the left-sided SMP in the region of the dorsal pancreatic bud (Fig. 3A). Thus, *Pitx2* expression is an indicator of left/right differences and shows that the left SMP expresses LR-specific genes. The homeobox-containing *Barx1* (Tissier-Seta et al., 1995), which has not previously been associated with LR asymmetry, also shows left-specific mesenchymal expression, substantiating the phenotypic differences between left and right mesenchyme at these early stages (Fig. 3B).

A previous report predicted that *Bapx1* has an early role in LR asymmetry as the gene is expressed in a side-specific

manner (Schneider et al., 1999). Paradoxically, *Bapx1* mesodermal expression is highest in the left LPM in chick and the right LPM in mouse. The asymmetric expression in mouse is initially detected at E8.0 and continues up to ~E9.5. We suggest that by E9.5, however, expression of *Bapx1* in the region of the pancreas is in the process of converting to left-sided expression. In the SMP, the expression is bilateral but the level is detectably higher on the left side (Fig. 3D). By E10.5, *Pitx2* is no longer detected (data not shown) while both *Barx1* (Fig. 3C) and *Bapx1* (Fig. 3E) are expressed at the extreme lateral domain of the SMP and in the underlying mesenchyme. Thus, there is a conversion in the sidedness of the *Bapx1* expression in the mouse and by E9.5 is in accordance with the expression pattern of the chick (Schneider et al., 1999).

To further investigate the link between SMP and the genetic LR asymmetry program, the spleno-pancreatic region in mice carrying the inversion of body turning (*inv*) mutation was analysed (Fig. 3F,G). Homozygous *inv* mutant embryos all show situs inversus (Yokoyama et al., 1993; Morgan et al., 1998; Watanabe et al., 2003) with both the spleen and pancreas developing on the right hand side. At E10.5 in the *inv/inv* embryos, the SMP is positioned on the opposite, right-hand side (Fig. 3G). The underlying spleno-pancreatic mesenchyme has grown to the right and the region is the mirror image of that in the wild type. These findings show that the maintenance of the SMP, and subsequent growth of the spleno-pancreatic mesenchyme, is a downstream consequence of the LR signalling pathway.
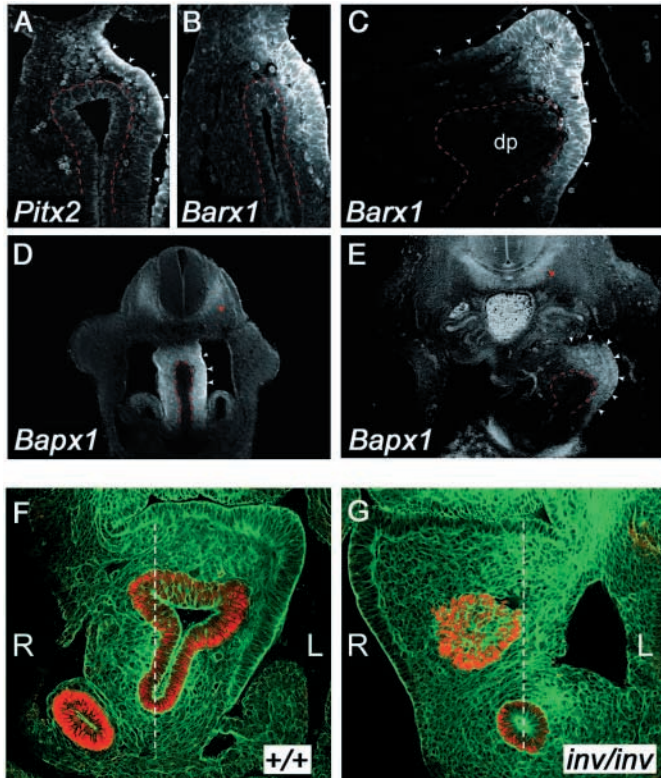
Fig. 3. The SMP is under the genetic control of LR signalling. Asymmetric expression of *Pitx2* (A) and *Barx1* (B) on the left-hand side of the mesenchyme and associated SMP (arrowheads) is detected at E9.5. At E10.5, *Pitx2* is not detected, while *Barx1* (arrowheads in C) is detected in the extreme lateral domain of the spleno-pancreatic mesenchyme. *Bapx1* expression is bilateral at E9.5 (D), but is higher on the leftward side (arrowheads) and is expressed in the left lateral domain at E10.5 (E). The pancreatic bud is outlined by a broken red line. (F,G) Immunohistochemical analysis of the *inv* mutant mouse using phalloidin (green) and PDX1 (red) antibody at E10.5. SMP formation is associated with the leftward-oriented spleno-pancreatic mesenchyme in wild type (F) and *inv* heterozygous embryos (data not shown), but develops in the opposite orientation in the homozygous mutant embryos (G).

Leftward pancreatic growth is dependent on the SMP

The *Dh* mutant embryo enables the examination of left lateral asymmetry in the absence of the SMP. In wild-type embryos at E10 the SMP and associated mesenchyme grows laterally (Fig. 4A). By contrast, in the *Dh/Dh* embryos the SMP remains undetectable and specific leftward growth of the mesenchyme is impaired (Fig. 4C). The mesenchyme is symmetrical and surrounds the dorsal pancreatic bud that remains situated along the embryonic midline. This arrangement persists at later stages and at E10.5 the pancreas is still observed at the midline (data not shown).

To investigate the possibility that the dorsal pancreas itself drives the leftward growth of the spleno-pancreatic mesenchyme, we analysed development of this region in *Fgf10* mutant mice. Loss of *Fgf10* expression has been reported previously to result in a severe disruption of pancreatic growth (Bhushan et al., 2001). Consistent with these findings, no

dorsal pancreatic tissue was detected at E10.5 in *Fgf10*$^{-/-}$ embryos following staining with the PDX1 antibody (Fig. 4G). In addition, no other endodermal-derived structures were found in the spleno-pancreatic mesenchyme and the duodenum remained at the embryonic midline. Despite this, the leftward growth of the spleno-pancreatic mesenchyme surrounded by the SMP occurred to approximately the same extent as the wild type (Fig. 4F,G).

Thus the *Dh* and *Fgf10*$^{-/-}$ mouse mutations provide two contrasting developmental conditions in which to examine leftward asymmetry. In the absence of the SMP, no leftward pancreatic growth was observed, whereas in the absence of the pancreatic bud, the SMP and underlying mesenchyme expand laterally. This suggests that asymmetry of the spleno-pancreatic mesenchyme and SMP is autonomous. The pancreatic endoderm in wild-type embryos is embedded in the left-sided mesenchyme and develops in close association with the SMP, but has no role, either physically or inductively, in the asymmetric growth of these structures.

Cells in the SMP proliferate at a high rate

As endodermal development is not required for asymmetric growth in the spleno-pancreatic region, we hypothesised that regional variations in cellular proliferation might be a key factor in the morphogenetic changes that characterise the development of the SMP and the underlying tissue. At E9.5, prior to overt LR asymmetry in this region, BrdU incorporation can be detected at high levels in both the left and right sides of the SMP (Fig. 5A). This pattern is reiterated at E9.75, during the initial stages of asymmetric morphogenesis. However, by this stage, regional differences in the BrdU-uptake pattern exist. Comparable levels of BrdU incorporation are observed in the left and right-sided SMP anterior to the dorsal pancreatic bud, with positive nuclei distributed evenly along the dorsoventral axis (Fig. 5B). However, in the region of the dorsal pancreatic bud, BrdU is detected in clusters of nuclei located around the apex of the outgrowing SMP (Fig. 5C).

By E10.5, distinct populations of cells can be readily discerned, enabling comparative analysis of BrdU incorporation throughout the stomach and pancreatic region. Wild-type embryos at ~ E10.5 were labelled with BrdU for 30 minutes and transverse sections through different regions of the gut (represented in Fig. 6B) were stained for BrdU incorporation (Fig. 6C-F). Mesothelial layers contiguous with the SMP in two embryonic regions were analysed; sections were taken anterior to the SMP in the region of the developing stomach (D in Fig. 6B) and in the region of the dorsal pancreatic bud that includes the SMP (E in Fig. 6B). Embryonic incorporation of BrdU was high throughout the embryo (Fig. 6C); ~50-55% of the cells labelled in the gut mesenchyme while in the gut endoderm about 35% of the cells labelled. Analysis of sections taken through the stomach show an associated thin mesothelial layer (white arrowheads in Fig. 6D) in which about 60% (±2%) of the cells incorporated BrdU (Fig. 6G). By comparison, the thickened SMP associated with the spleno-pancreatic region shows ~77% (±3%) incorporation (Fig. 6G). The mesothelium that lines the lateral coelomic walls (blue arrowheads in Fig. 6D-F) incorporates BrdU at a rate of about 50% (Fig. 6G) or less. Posterior to the pancreas (F in Fig. 6B) the gut mesoderm is no longer flanked by the SMP and the BrdU uptake in the mesoderm and associated
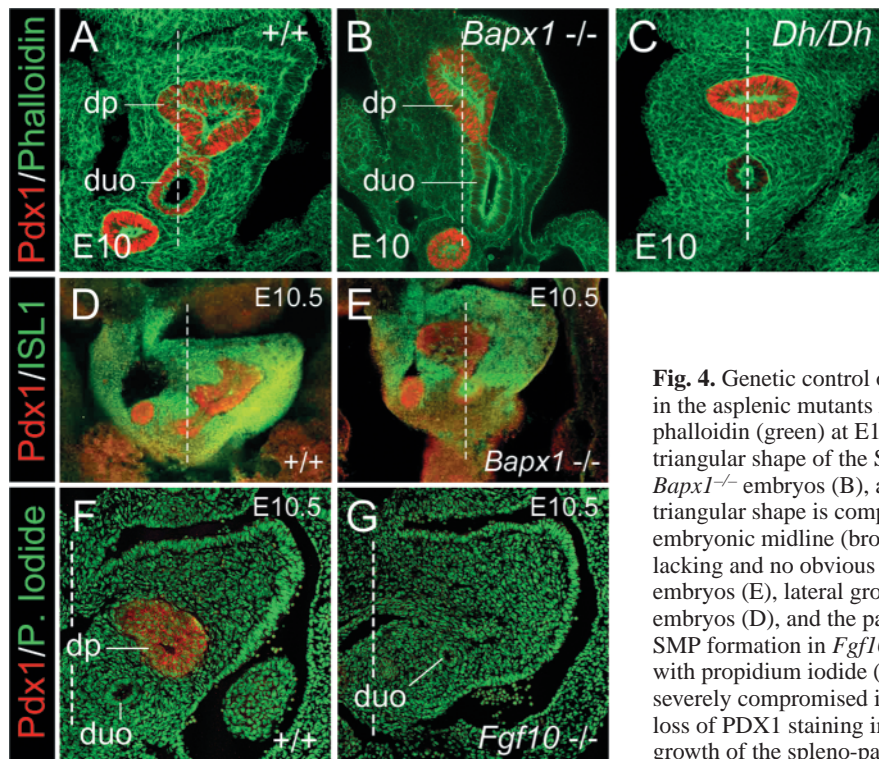
Fig. 4. Genetic control of SMP formation. (A-C) Analysis of SMP formation in the asplenic mutants *Dh* and *Bapx1*^{−/−}, using PDX1 antibody (red) and phalloidin (green) at E10. (A) Wild-type embryos show the characteristic triangular shape of the SMP and spleno-pancreatic mesenchyme, whereas in *Bapx1*^{−/−} embryos (B), although the mesenchyme grows to the left, the triangular shape is compromised and the pancreatic bud remains near the embryonic midline (broken line). In the *Dh/Dh* embryos (C), the SMP is lacking and no obvious leftward growth is detected. At E10.5 in *Bapx1*^{−/−} embryos (E), lateral growth occurs but to a lesser extent than in wild-type embryos (D), and the pancreas remains at the midline. (F,G) Analysis of SMP formation in *Fgf10*^{−/−} embryos following staining for PDX1 (red), and with propidium iodide (green). Development of the pancreatic endoderm is severely compromised in the *Fgf10*^{−/−} embryos, as revealed by an apparent loss of PDX1 staining in the mutant embryos (G). Despite this, leftward growth of the spleno-pancreatic mesenchyme and SMP occurs normally (G).

mesothelium are appreciably lower than the SMP (<50% incorporation) (Fig. 6F). Thus, the SMP shows a higher rate of BrdU incorporation when compared with the mesothelia in other regions of the embryo and the mesenchyme underlying the SMP. We suggest that the SMP has a role in the left lateral disposition of the mesenchyme that is crucial for both the formation of the spleen and the asymmetric morphogenesis of the pancreas. The asymmetric growth is driven by the high rate of cellular proliferation in the SMP.

The *Bapx1* gene regulates functions of the SMP

Similar to the *Dh* mutant, the *Bapx1*^{−/−} embryos are asplenic. Mice homozygous for the *Bapx1* mutation were examined at the early stages of spleno-pancreatic outgrowth, E10 and E10.5 (Fig. 6B,E, respectively). In contrast to *Dh* embryos, the *Bapx1* mutants retain the left SMP throughout these stages. The spleno-pancreatic mesenchyme of the *Bapx1* mutant expands laterally but lags behind that of wild type and the characteristic triangular shape of the wild-type SMP is compromised. Most significantly, the dorsal pancreatic bud of the *Bapx1* mutant

does not grow laterally but remains positioned along the embryonic midline.

To investigate the effect that the early malposition of the pancreas has on later development, we examined both the *Dh* and *Bapx1* mutants at a later stage in organogenesis. Whole-mount, three-dimensional analysis of organ position by optical projection tomography (OPT) (Sharpe et al., 2002) revealed a significant change in the orientation (Fig. 7A-C). At E13.5 in both *Dh* and *Bapx1* mutants, the pancreas is growing along a different embryonic axis. In wild-type embryos, the dorsal pancreas grows laterally in a plane that is nearly perpendicular to the stomach (Fig. 7A). In both mutants, the dorsal pancreas grows along embryonic axes that are close to parallel with the stomach. In the *Bapx1* embryos, the pancreas grows along the lateral wall of the stomach (Fig. 7C), whereas in the *Dh* embryo the pancreas is oriented ventrally to the stomach (Fig. 7B).

The SMP is an early source of growth factors

A number of genes predicted to be relevant to embryonic growth were assayed for expression in the spleno-pancreatic
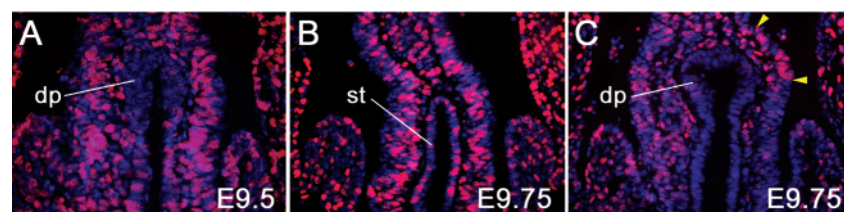

Fig. 5. Analysis of BrdU incorporation in the developing SMP. (A-C) At E9.5, BrdU incorporation is comparable on both sides of the bilateral SMP (A), with proliferating cells evenly distributed along the DV axis. This pattern is reiterated at E9.75 in sections cut through the posterior stomach (B). The initial outgrowth of the left-sided SMP in the region of the pancreatic bud is accompanied by a clustering of BrdU-positive nuclei at the prospective apex (yellow arrowheads in C). dp, dorsal pancreatic bud; st, posterior stomach.
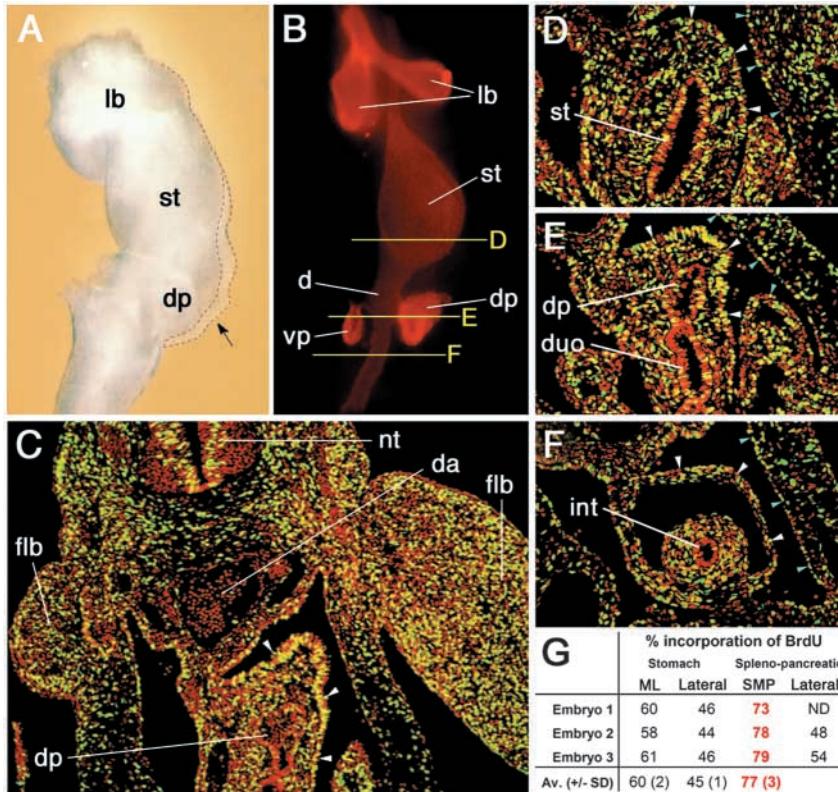
Fig. 6. Rate of cellular proliferation is high in the SMP. (A) Bright-field illumination of an E10.5 embryonic gut showing the mesenchyme that surrounds the endoderm and the refractive properties of the SMP (broken lines). (B) The gut endoderm present in A is highlighted using an antibody specific to the endodermal-specific marker HNF3β. (D-F) Representative sections through the spleno-pancreatic region (E), the stomach region (D) and the posterior gut region (F) at positions indicated in B. Analysis of BrdU incorporation in the developing embryonic gut; cellular incorporation of BrdU is indicated by green (low level) and yellow (high level) stains. Percent incorporation of BrdU from these regions is quantified in G; the high incorporation in the SMP is highlighted in red. (C) Transverse section through the region of dorsal pancreatic bud to give an overview of BrdU incorporation in tissues. There is high incorporation in the SMP (white arrowheads) relative to other tissues. (D) The thin mesothelial layer (white arrowheads) associated with the stomach (ML in G) and the lateral coelomic mesothelium (blue arrowheads) (lateral in G), and (E) the SMP (white arrowheads) and lateral coelomic mesothelium (blue arrowheads) (lateral in G) were also analysed. Sections through the gut posterior to the pancreatic bud (F) were also analysed but are not included in G. d, duodenum; da, doral aorta; dp dorsal pancreas; flb, forelimb bud; lb, lung buds; nt, neural tube; st, stomach; vp, ventral pancreatic bud.

region. Using RT-PCR, 60 genes, including eight Fgfs were examined. *Fgf 9*, *Fgf10*, *Fgf11* and *Fgf13* were found to be expressed in the spleno-pancreatic region at E10.5. Expression of these genes was analysed by in situ hybridisation in transverse sections. Whereas *Fgf11* and *Fgf13* were expressed at detectable levels throughout the mesenchyme (data not shown), both *Fgf9* and *Fgf10* were restricted to domains on the left-side encompassing the SMP. *Fgf9* is expressed most highly in the dorsal rim, including the lateral tip of the SMP and extends into the underlying mesenchyme (Fig. 8B). *Fgf10* was previously reported to be expressed in the pancreatic mesenchyme (Bhushan et al., 2001). Here, we show that, more specifically, *Fgf10* is expressed in the ventral region of the SMP and in the associated underlying mesenchyme (Fig. 8A). In addition, we detected expression of *Fgfr3* in the extreme tip of the SMP (Fig. 8C). FGFR3 is a high affinity receptor for FGF9 and its expression in this region therefore suggests a model of *Fgf9* induced outgrowth of the SMP.

In the *Dh* mutant, in the absence of the SMP, expression of *Fgf9* and *Fgf10* were both substantially reduced (data not shown). In the *Bapx1* mutant, *Fgf10* expression in the SMP was dramatically downregulated (Fig. 8D) although there was persistent expression in the mesenchyme surrounding the pancreatic bud. *Fgf9* expression was reduced but still detectable (data not shown). The SMP therefore appears to be a source of the secreted factors *Fgf9* and *Fgf10*. In the absence of the SMP, or following perturbation in its development, expression of Fgfs is compromised. Our findings suggest that *Bapx1* is upstream of, and required for, *Fgf10* expression in the SMP.

Discussion

Association of spleen and pancreas morphogenesis

The spleen originates in the mesenchyme that is located posterior to the stomach and adjacent to the dorsal pancreas.
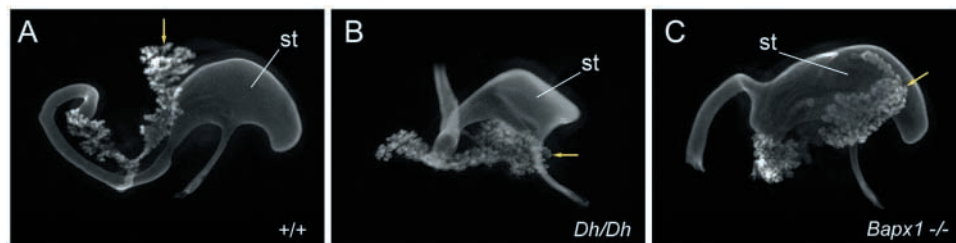

Fig. 7. Location of the pancreas is misplaced in the *Bapx1* and *Dh* mutants. Three-dimensional examination of the region around the stomach at E13.5 using OPT. The endoderm is highlighted using an antibody to E-cadherin. The position of the pancreas is indicated with a yellow arrow. The wild-type embryonic gut shows the pancreas growing along an axis perpendicular to the duodenum (A). In both *Dh/Dh* (B) and *Bapx1*^{−/−} (C) embryos, the pancreas grows along the same axis as the stomach.
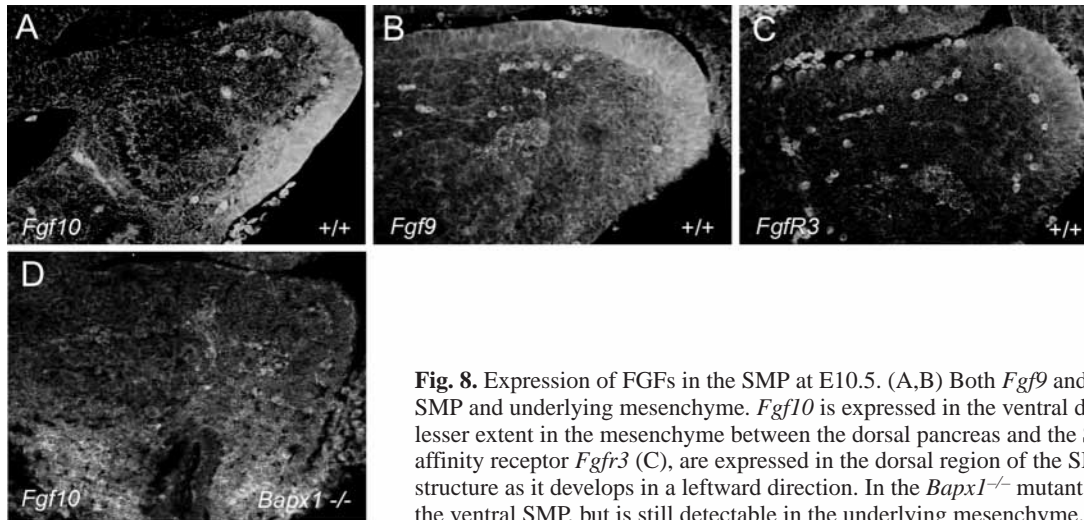
Fig. 8. Expression of FGFs in the SMP at E10.5. (A,B) Both *Fgf9* and *Fgf10* are expressed in the SMP and underlying mesenchyme. *Fgf10* is expressed in the ventral domain of the SMP and to a lesser extent in the mesenchyme between the dorsal pancreas and the SMP (A). *Fgf9* (B), and its high affinity receptor *Fgfr3* (C), are expressed in the dorsal region of the SMP and towards the tip of this structure as it develops in a leftward direction. In the *Bapx1*$^{-/-}$ mutant (D), *Fgf10* is downregulated in the ventral SMP, but is still detectable in the underlying mesenchyme.

The leftward expansion of the mesenchyme supplies the tissue in which the spleen forms and in which the leftward growing dorsal pancreas resides. Thus, initiation of splenogenesis and leftward pancreatic growth are closely linked. The close proximity in early development is consistent with studies by Patterson et al. (Patterson et al., 2000) that showed a close association between spleen and pancreatic laterality in experiments designed to randomise the embryonic LR axis. These indicated that the location of the spleen strongly correlated with the position of the pancreas and not other organs, such as the heart. This idea is further supported by the observation that some individuals with heterotaxy that includes spleen malformations have no detectable heart defects (Debich et al., 1990), and by analyses of the *iv* mutant mice, where no consistent relationship of heart and spleen laterality defects was shown (Seo et al., 1992).

Therefore, while determination of LR asymmetry is a general developmental mechanism, it appears that LR information is interpreted differently by each organ primordia. We suggest that *Bapx1* and *Dh* mutants are good models for heterotaxy syndromes that include asplenia (double right isomerism). Neither mouse mutant shows cardiac, lung or liver malformations (Green, 1967; Herbrand et al., 2002) (data not shown) and, therefore, they provide insights into laterality disorders in which only a restricted number of organ systems are affected. We propose that the developmental mechanism that drives asymmetric organ morphogenesis in spleen and pancreas differs from that responsible for lobation of the lung and morphogenesis of the heart tube and that it is dependent on a mesodermal-derived structure, the SMP.

Mechanism for spleno-pancreatic LR asymmetry

The SMP is central to our model for left lateral specific morphogenesis of the spleen and the pancreas (Fig. 9). The process of LR asymmetry is divided into four steps (Hamada et al., 2002). The first three steps are responsible for transferring an initial breaking of symmetry from near the node (step 1), to the LPM (step 2) with the subsequent asymmetric expression of TGFβ-related molecules (step 3). Less clearly understood is the fourth step, which is the relay of information from the LMP to the organ primordia. The SMP appears to be

an important element in this final step and in the spleno-pancreatic region may be the primary target for the LR positional information.

At the earliest developmental stages examined, E9.5, the SMP is bilateral and flanks the midline-positioned dorsal pancreatic bud. There is no intervening space and few mesenchymal cells are found in between the SMP and the pancreatic endoderm. The left side of the SMP is specified at this early stage, as shown by the side-specific expression of both *Pitx2* and *Barx1*. The LR positional information is subsequently interpreted as maintenance of the left lateral SMP and loss of the right. The *inv* mutant mouse confirms that the unilateral maintenance of the SMP is under the genetic control of the LR asymmetry cascade.

The *Dh* mutation underscores the distinctive structural nature of the SMP. The mutation operates early in embryogenesis to disrupt the bilateral organised, columnar structure. We suggest that the highly organised structure of the SMP plays a significant role in morphogenesis. In several cases, disruption of the structural integrity of embryonic epithelia has resulted in profound perturbations in subsequent morphogenesis. A similar highly organised epithelia, which is derived from the lateral plate mesoderm, is key to the process of gut looping in zebrafish (Horne-Badovinac et al., 2003). Genetic disruption of the cellular organisation results in the lack of morphogenesis. Thus, the structural context of the SMP may be crucial to lateral outgrowth and we speculate that the structure may provide a rigid tissue layer for guidance. Accordingly, the SMP is characterised by the accumulation of f-actin at the apical surfaces as detected by phalloidin staining. Localisation of actin filaments to the apical surface of columnar epithelial cells has been described in a number of organisms (reviewed by Jacinto and Baum, 2003) and networks of actin filaments have recently been shown to play a crucial role during the morphogenesis of the pharyngeal pouches by directing expansion along specific axes (Quinlan et al., 2004).

A second characteristic of the SMP important for our model of lateral outgrowth is the high rate of cellular proliferation in this tissue layer. Outgrowth of the spleno-pancreatic mesenchyme and SMP is autonomous and entails no discernible input from the pancreatic endoderm. Proliferation
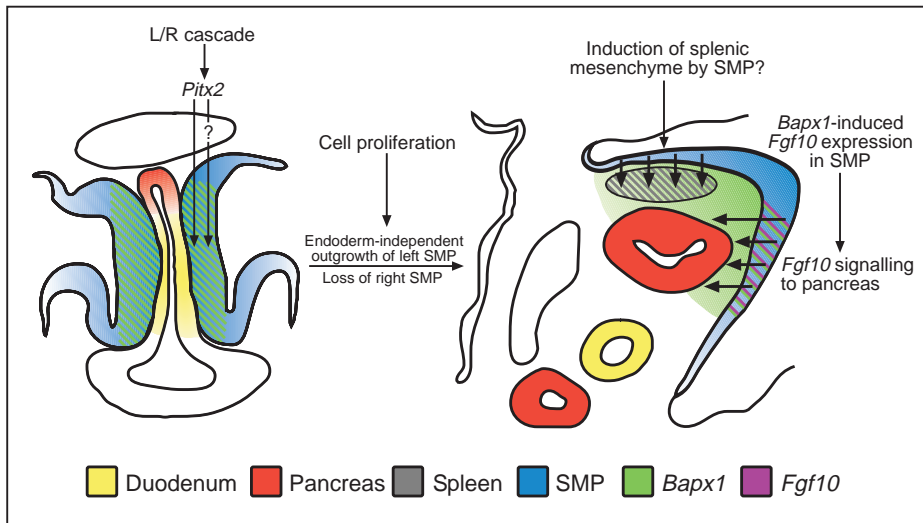
Fig. 9. Model for asymmetry in the spleno-pancreatic region. The model describes the major events that occur in lateral organ morphogenesis. The left SMP is under the influence of the LR genetic cascade and is maintained whereas the right SMP is lost. Cell proliferation in the SMP appears to be the motive force in the lateral growth that occurs in the SMP between E9.5 and E10.5 of development. During this period, the population of mesenchymal cells underlying the SMP expands considerably and the dorsal region is induced (perhaps by the associated SMP) to become spleen. Outgrowth of the spleno-pancreatic region is compromised by loss of *Bapx1*, as is *Fgf10* expression in the ventral SMP. The high expression of *Fgf10* from the SMP may be the chemotactic factor to which the dorsal pancreas responds.

within this cellular layer appears to be the motive force for driving lateral growth. We suggest that within a highly organised cellular framework proliferation within the SMP drives rapid, disproportionate expansion of this tissue layer behind which the mesenchyme, perhaps passively, populates.

Since the description of asymmetric *Bapx1* expression during early mouse and chick embryogenesis, it has been postulated that *Bapx1* may have a role to play in the establishment of LR asymmetry (Rodriguez Esteban et al., 1999; Schneider et al., 1999). The exact nature of involvement of *Bapx1* in this process has, however, remained undetermined. Based on the reported expression patterns, it is tempting to speculate that *Bapx1* could be playing a role in the early establishment of positional information. Paradoxically, the laterality of the expression is not conserved in evolution (Schneider et al., 1999). Our observations suggest that *Bapx1* in mouse loses side-specific expression at a stage (E9.5) before any phenotype is observed in the *Bapx1*−/− spleno-pancreatic region. Expression of the *Bapx1* gene persists after that of *Pitx2*, a late stage gene in the LR cascade. The defects observed in the *Bapx1*−/− embryos reported here relate specifically to the process of morphogenesis and are in agreement with a role for *Bapx1* in the translation of positional information into asymmetric morphogenesis. Thus, we suggest that *Bapx1* has a key role in the process of linking organ morphogenesis to events that define LR positional information.

Two phenotypic consequences result from the lack of *Bapx1* expression. The first is the lateral growth of the spleno-pancreatic mesenchyme is reduced. The second is the downregulation of *Fgf10* expression. *Bapx1* is expressed in the ventral region of the SMP overlapping the *Fgf10* domain, consistent with *Bapx1* as a regulator of *Fgf10* expression. FGF10 itself is responsible for early pancreatic growth (Bhushan et al., 2001), and in its absence the pancreas does not progress beyond the early bud stage. *Fgf10* is expressed in the SMP and neighbouring mesenchyme at a source distal to the pancreatic bud. FGF10 is a known chemotactic factor in lung development (Weaver et al., 2000). We suggest that, in addition to its role in maintaining proliferation in the pancreatic endoderm, a role for FGF10 is to promote leftward pancreatic growth toward the source of high FGF10 production, thus resulting in the initial pancreatic asymmetry.

Organ morphogenesis in other vertebrates

Recent analysis in the developing zebrafish has addressed the question of gut asymmetry (Horne-Badovinac et al., 2003). The digestive organs originate from a solid rod of endodermal cells and the first leftward bend arises from a morphogenetic process known as gut looping. The tissue layer that appears responsible for the initial mechanism in gut looping is the LPM. The relationship of the LPM in zebrafish and the SMP in mouse is not clear. The SMP is derived from the mesoderm of the lateral plate and both the LPM described in fish and SMP described here, are similar epithelial layers composed of columnar cells. However the mechanism of LR organogenesis in which these embryonic tissues participate is different. In the zebrafish, the lateral plate mesoderm (LPM) flanks the gut tube on the left and right sides, and by coordinated tissue migration drives the initial asymmetry of the gut tube by a pushing mechanism (Horne-Badovinac et al., 2003). In the mouse embryo, the mechanism relies on the gut mesoderm, in which leftward expansion of the mesenchyme and lateral morphogenesis of the pancreas appears to follow growth of the SMP.

Is it possible that organ morphogenesis shows appreciable species differences? Many of the genes that specify organ identity and cellular differentiation are highly conserved. However species have different requirements in the gut depending on food source and diet, and in vertebrates separated by such an evolutionary distance as mammals and fish some differences may be expected to occur. An important example is that the zebrafish has no stomach (Smith, 1982). In addition, some endodermal components of the gut are generated differently. It is clear, for example, that fish and mammals generate the pancreatic rudiment in a different manner. In fish, the pancreas does not bud from the endodermal gut tube, but instead is derived from endoderm that is peripheral to the intestine (Wallace and Pack, 2003). The mechanisms for organ morphogenesis must therefore respond to these different requirements and may underlie species differences.

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