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The dynamics of spleen morphogenesis

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ABSTRACT

The mammalian spleen has important functions in immunity and haematopoiesis but little is known about the events that occur during its early embryonic development. Here we analyse the origin of the cells that gives rise to the splenic mesenchyme and the process by which the precursors assume their position along the left lateral side of the stomach. We report a highly conserved regulatory element that regulates the *Nkx2-5* gene throughout early spleen development. A transgenic mouse line carrying this element driving a reporter gene was used to show that morphogenesis of the spleen initiates bilaterally and posterior to the stomach, before the splenic precursors grow preferentially leftward. In addition the transgenic line was used in an organ culture system to track spleen precursor cells during development. Spleen cells were shown to move from the posterior mesenchyme and track along the left side of the stomach. Removal of tissue from the anterior stomach resulted in splenic cells randomly scattering suggesting a guidance role for the anterior stomach. Using a mouse line carrying a conditional Cre recombinase to mark early precursor cell populations, the spleen was found to derive from posterior mesenchyme distinct from the closely adjacent stomach mesenchyme.

Introduction

The mammalian spleen is located on the left side of the body, between the diaphragm and the fundus of the stomach, and has important functions in immunity and haematopoiesis (Mebius and Kraal, 2005). Asplenia, the absence of the spleen, is regarded as bilateral right-handedness and can be found in isolation or, more commonly, in association with other laterality defects. In heterotaxy syndrome the visceral situs along the left–right axis is abnormal, resulting in cardiovascular abnormalities and a range of spleen defects; these include asplenia, polysplenia (spleen tissue on both sides of the body–bilateral left-handedness–or multiple splenuli on one side), or a right-sided spleen (Bartram et al., 2005). The risk of sepsis is 10- to 20-fold higher in asplenic individuals and can result in death especially in young children (Brendolan et al., 2007).

To understand how spleen malformations arise, it is essential to first understand how splenic precursors normally grow out to form the spleen, how it develops at the correct location on the left side of the stomach, and what signals are involved. The complete process of spleen development has not yet been characterised, as concluded in a recent review (Brendolan et al., 2007), and the key questions of what processes underlie spleen patterning, morphogenesis and expansion remain unanswered.

During embryonic development, splenic precursors can be first detected at ~E10.5–11.0 as a mesenchymal condensation within the left side of the dorsal mesogastrium, adjacent to the stomach and dorsal pancreas (Thiel and Downey, 1921; Green, 1967; Brendolan et al., 2005, 2007). This putative splenic mesenchyme underlies the splanchnic mesodermal plate (SMP), an organised transient structure which plays a key role in spleen development (Hecksher-Sorensen et al., 2004). The SMP expresses *Bapx1* (*Nkx3-2*) and directs the leftward outgrowth of the underlying spleno-pancreatic mesenchyme (Hecksher-Sorensen et al., 2004).

The putative splenic mesenchyme is closely associated with the dorsal pancreatic mesenchyme and expresses a number of markers expressed in later spleen development, implicating it as the source of splenic precursor cells. *Bapx1*, *Nkx2-5*, *Tlx1* (*Hox11*), *Pod1* (*Capsulin*), *Wt1*, and *Pbx1* are all expressed in both the condensed mesenchyme and later in the developing spleen (Hecksher-Sorensen et al., 2004; Roberts et al., 1994; Lu et al., 1998; Rackley et al., 1993; Brendolan et al., 2005). Loss of most of these genes has been shown to result in asplenia (Lettice et al., 1999; Roberts et al., 1994; Lu et al., 2000; Herzer et al., 1999; Brendolan et al., 2005); it has not, however, been possible to analyse the requirement for *Nkx2-5*, as loss of this gene proves lethal at E9.0–10.0 before spleen development is detectable (Lyons et al., 1995). In *Xenopus*, *XNkx2-5* appears to be the earliest marker of splenic precursors (Patterson et al., 2000). *XNkx2-5* expression is

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initially present on both sides of the embryo, indicating a preliminary bilateral existence of splenic precursors. The left-side pool then preferentially develops into the spleen. The gene most recently implicated in spleen morphogenesis is *Barx1*, loss of which causes hypoplasia and malpositioning of the developing spleen (Kim et al., 2007).

Bapx1 is one of the early markers of spleen development. The putative splenic mesenchyme fails to condense in *Bapx1* null embryos, instead persisting as loosely organised cells adjacent to the dorsal pancreatic bud (Asayesh et al., 2006). The splenic and pancreatic mesenchymes remain inappropriately juxtaposed in these embryos, demonstrating that highly coordinated interactions between these tissues are necessary for their correct development (Asayesh et al., 2006). *Bapx1*$^{-/-}$ embryos exhibit asplenia and lack *Hox11* expression at E12 (Lettice et al., 1999).

In this paper we focus on the early stages of spleen development and show that splenic markers are initially expressed symmetrically, posterior to the putative stomach. These markers become asymmetrically localised concomitant with the first signs of leftward organogenesis; their expression domains then expand leftward and anteriorly towards the stomach. Once in contact the outgrowth of spleen precursor cells occurs in response to a signal from the anterior part of the stomach. Spleen morphogenesis is therefore a dynamic process in which precursors become limited to the left side posterior to the stomach rudiment and move anteriorly until spread across the length of the stomach.

Material and methods

Generation of Nkx2-5 Gut Regulatory Sequence (NGRS)-LacZ line and Nkx2-5 in situ hybridisation

A 1956 bp fragment upstream of *Nkx2-5* was isolated (*Nkx2-5* Gut Regulatory Sequence: *NGRS*) from genomic C57BL/6 mouse DNA and ligated into p1230, a *LacZ* reporter construct containing a heterologous *ß-globin* promoter (based on pBGZ40 (Yee and Rigby, 1993)). Transgenic mice were made as previously described (Lettice et al., 2003). The resulting line is referred to in this paper as *NGRS-LacZ*.

Nkx2-5 expression was analysed by *in situ* hybridisation, using a DIG-labelled antisense RNA probe reverse transcribed from a PCR product template (F: ACTTGAA-CACCGTGCAGAGTCC, R: *TAATACGACTCACTATAGGG*GTGTGG AATCCGTCGAAAGTGC). Whole mount embryos were hybridised according to standard procedures (Hecksher-Sorensen et al., 2004). Standard detection was performed using NBT/BCIP and fluorescent detection with Fast Red (both Roche Diagnostics, Mannheim, Germany). Stained embryos were embedded in agarose, sectioned on a vibratome, and the signal examined using either light (NBT/BCIP) or confocal microscopy (Fast Red).

Tissue collection for gut culture and LacZ analysis

Embryonic guts (including the stomach, spleno-pancreatic mesenchyme, intestine, and lung buds) to be cultured were dissected in DMEM (Gibco, Invitrogen Corporation, Paisley, UK), supplemented with 10% foetal calf serum (Hyclone, Perbio, Cramlington, UK) and 1% penicillin/streptomycin (Sigma, Dorset, UK), from *NGRS-LacZ*$^{+}$, *Bapx1*$^{+/-}$*NGRS-LacZ*$^{+}$, or *Bapx1*$^{-/-}$*NGRS-LacZ*$^{+}$ mouse embryos (Lettice et al., 1999). Tungsten needles were used to remove a posterior part of the guts (n=19) or part of the anterior stomach (n=13).

The dissected guts were transferred to a 24-well plate shortly after dissection and covered with a thin layer of 20 µl growth factor reduced Matrigel (BD Biosciences, Bedford, UK). The guts were imaged and the Matrigel was allowed to set for 1 h at 37°C at 5% CO_2; subsequently 200 µl supplemented DMEM was added and the cultures grown for 48 h at 37°C and 5% CO_2. Explants were imaged again at the end of the culture period.

Cultured guts were fixed in 4% paraformaldehyde (in PBS) for 15 min and X-gal stained overnight. Non-cultured embryos and embryonic guts were dissected in cold PBS prior to fixing and staining.

Optical projection tomography (OPT)

OPT analysis of *NGRS-LacZ* expression and gut anatomy was performed as described (Sharpe et al., 2002). Image reconstructions were performed using the Amira 4.1 software package (Mercury Computer Systems).

Clonal analysis

The R26R-RERT mouse line was used for clonal analysis; this is a cross between lines carrying the R26R reporter (floxed *LacZ* inserted in the *ROSA26* locus) (Soriano, 1999) and RERT (*IRES-CreERT2* insertion in the *Pol2* gene) (Guerra et al., 2003) transgenes.

Tamoxifen (4-hydroxytamoxifen; Sigma, Madrid, Spain) was resuspended in corn oil (Sigma) at 0.5 g/l and sonicated 5 min before injection. Tamoxifen concentrations ranging from 1 to 500 µg/g female body weight were injected intraperitoneally into pregnant females at E7.5–9.0. Recombination events are highly dependent on tamoxifen administration; previously the background (recombination events without induction) was determined to be very low and those events detected were predicted to occur at late stages in development (Arques et al., 2007). Embryos were collected at E11.5–E14.5 and fixed in 1.25% glutaraldehyde in PBS for 30 min and stained overnight in X-gal stain solution according to standard procedures (Arques et al., 2007). Transparency for imaging was increased by graded solutions of glycerol up to 80%.

Results

Nkx2-5 expression marks early spleen development

Morphologically, the first overt manifestation of the spleen occurs at approximately E11.5 as condensed mesenchyme located lateral to the stomach and associated with the stomach mesenchyme. Previous studies have shown that spleen markers are expressed as early as E10.5 (review Brendolan et al., 2007) within the mesenchyme associated with the pancreatic bud and lying posterior to the stomach (Hecksher-Sorensen et al., 2004; Asayesh et al., 2006). These observations raise several possible models for the development of the spleen. The first notion is that splenic mesenchyme derives solely from this early posterior tissue. The spleen subsequently forms from this mesenchyme which moves anteriorly along the left lateral aspect of the developing stomach. An alternative model incorporates the left lateral stomach mesenchyme and suggests a contribution from this tissue to the spleen such that the spleen initiates posterior to the stomach but local stomach mesenchyme contributes at subsequent stages. To investigate the origin of the splenic mesenchyme and distinguish these two possibilities a putative early spleen marker was used to analyse the initial stages of spleen development.

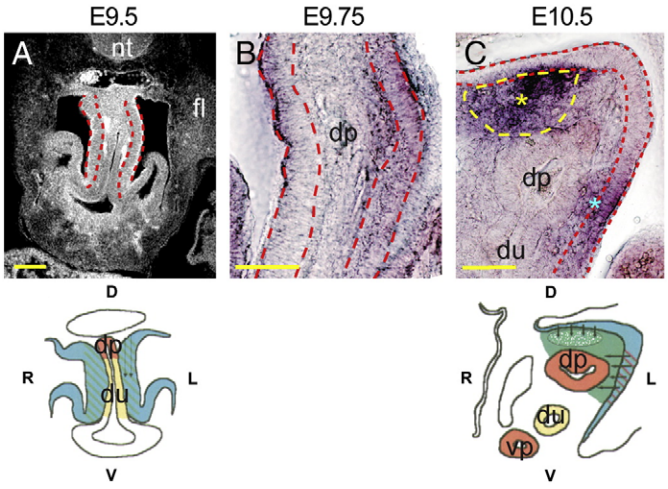

Fig. 1. The spleen marker *Nkx2-5* is expressed in the splanchnic mesodermal plate (SMP) at E9.5–10.5. *In situ* hybridisation showed that *Nkx2-5* is expressed bilaterally throughout the SMP (bounded by dashed red line) and the nascent underlying mesenchyme at E9.5 (A), but becomes increasingly restricted to the left side from E9.75 (B). *Nkx2-5* expression is localised to two domains at E10.5 (C): one encompassing the ventral mesenchyme and SMP (blue asterisk at centre), and one in the dorsal mesenchyme underlying the SMP (yellow asterisk at centre). This latter domain overlaps with the expression of other splenic markers (Hecksher-Sorenesen et al., 2004) and is proposed to be the putative splenic mesenchyme (outlined with yellow dashed line). Images are of vibratome sections through whole mount embryos, at the following thicknesses: (A) 70 µm, (B) 150 µm, (C) 80 µm. The lower level expression pattern in panel A was visualised fluorescently and imaged by confocal microscopy to provide sufficient detail; those in panels B and C were detected using NBT/BCIP and standard light microscopy. The yellow scale bars represent 100 µm. Schematics of E9.5 and E10.5 guts, with the SMP shown in blue, are provided underneath the photographs (adapted from those in Hecksher-Sorenesen et al., 2004). Abbreviations: D: dorsal; dp: dorsal pancreatic bud; du: duodenum; fl: forelimb; L: left; nt: neural tube; R: right; V: ventral; vp: ventral pancreatic bud.

Nkx2-5 is expressed in the developing spleen in mouse (Lints et al., 1993; Kasahara et al., 1998; Moses et al., 2001; Hecksher-Sorensen et al., 2004) but is reported to be the earliest spleen marker in *Xenopus* (Patterson et al., 2000). *In situ* hybridisation analysis of *Nkx2-5* expression at E9.5 and E10.5 showed that *Nkx2-5* is indeed expressed early in mouse gut development, in the SMP and underlying mesenchyme. *Nkx2-5* is expressed throughout the SMP and nascent mesenchyme at E9.5 (Fig. 1A), on both sides of the embryo. Expression becomes restricted to the left side from E9.75 (Fig. 1B), and by E10.5 is wholly left-sided and localised to two domains (Fig. 1C); the dorsal expression domain overlaps that of a number of other splenic markers and corresponds to the putative splenic mesenchyme (Hecksher-Sorensen et al., 2004). The bilateral expression in the SMP at E9.5 corresponds with the initially bilateral origin of *XNkx2-5*-expressing spleen precursors in *Xenopus* (Patterson et al., 2000).

An Nkx2-5 regulatory element labels spleen precursors

To facilitate investigation of early spleen morphogenesis we generated a reporter gene that marks spleen tissue. The *Nkx2-5* expression patterns described in the previous section suggested that *Nkx2-5* could provide such a marker. A number of regulatory regions have been identified in the *Nkx2-5* genomic neighbourhood (Schwartz and Olson, 1999). One such region lies between ~3.5 and ~1.6 kb

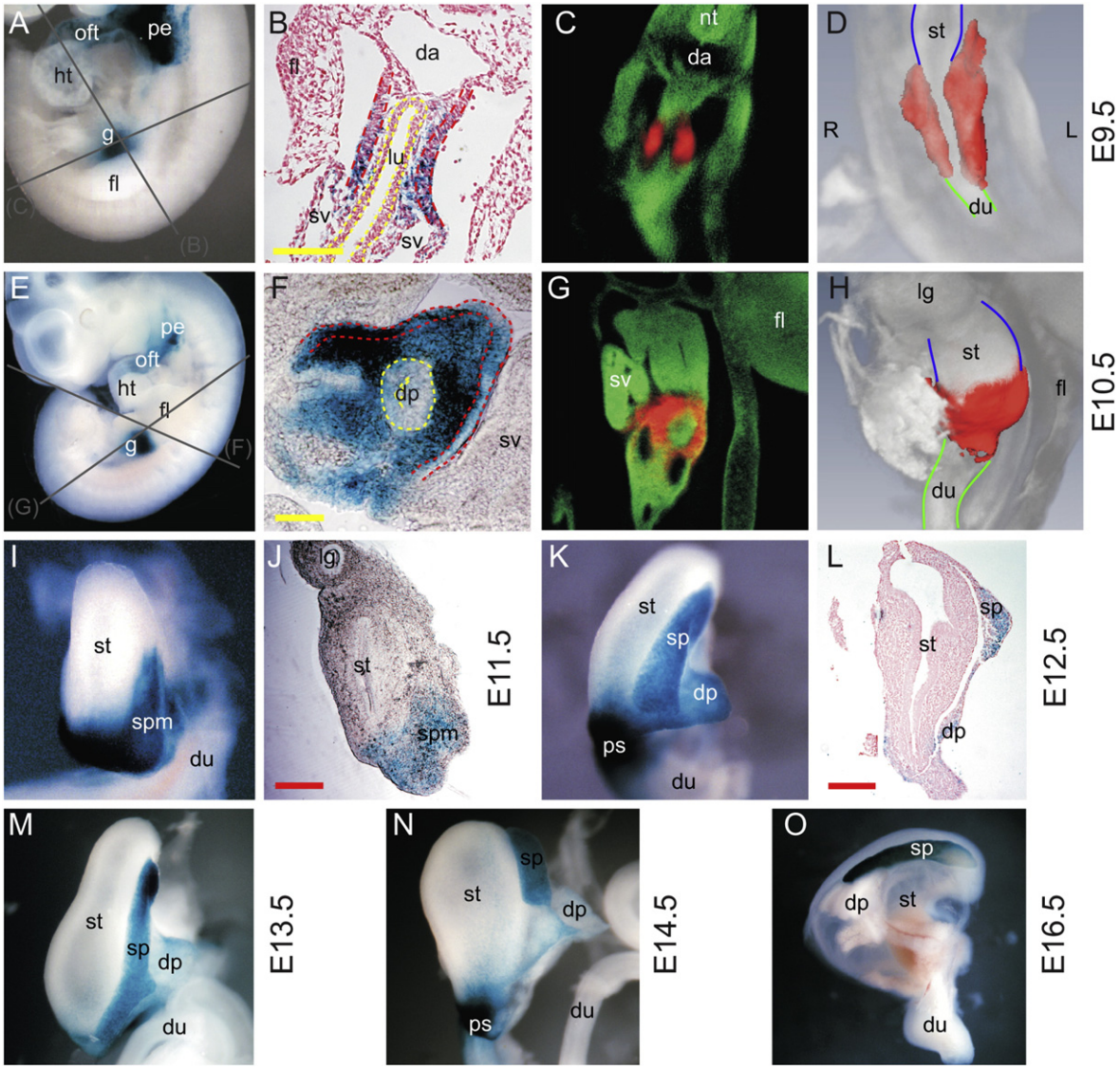

Fig. 2. The Nkx2-5 gut regulatory sequence (NGRS) drives *LacZ* expression throughout spleen development. *NGRS-LacZ* expression was detected by X-gal staining and is shown at E9.5 (A–D), E10.5 (E–H), E11.5 (I, J), E12.5 (K, L), E13.5 (M), E14.5 (N), and E16.5 (O). *LacZ* expression was strong in the gut (g) of whole E9.5 (A) and E10.5 (E) embryos, and was also detected in the pharyngeal endoderm (pe) and weakly in the outflow tract (oft). A 9 μm microtome section through an E9.5 embryo (B) demonstrates the initially bilateral expression of *NGRS-LacZ* in the SMP and underlying mesenchyme. By E10.5 expression is predominantly left-sided, in the SMP and underlying mesenchyme, as shown in the 100 μm vibratome section through an E10.5 embryo (F). Expression visualised by OPT is shown in red in the virtual sections through E9.5 (C) and E10.5 (G) embryos, and in the static images (D, H) from three-dimensional reconstructions–created using the Amira software–which show the extent of *NGRS-LacZ* expression along the anterior–posterior axis (anterior is at the top of the image, posterior at the bottom). The E11.5 X-gal staining pattern is shown in a whole mount gut (I), viewed from above the greater curvature of the stomach, and in a 30 μm vibratome section through an E11.5 gut (J), showing the spleen rudiment associated with the posterior half of the stomach. Expression at E12.5 is shown in a whole mount gut (K) viewed from above the greater curvature and in a 7 μm microtome section (L) showing staining adjacent to the left-lateral wall of the stomach. *NGRS-LacZ* expression continues in the spleen of E13.5 (M) and E14.5 guts (N), viewed from the greater curvature, and in a dorsal view of an E16.5 gut (O). Sectioning angles are shown as grey lines in panels A and E. The SMP is outlined with a red dashed line, the gut endoderm with a yellow dashed line in panels B and F. In panels D and H the stomach is outlined in blue and the duodenum in green. Microtome sections were counterstained with Nuclear Fast Red. Yellow scale bars represent 100 μm; red bars represent 200 μm. Abbreviations: da: dorsal aorta; dp: dorsal pancreas; du: duodenum; fl: forelimb; g: gut; ht: heart; L: left; lu: lumen of gut tube; lg: lung bud; nt: neural tube; oft: outflow tract; pe: pharyngeal endoderm; R: right; sp: spleen; spm: spleno-pancreatic mesenchyme; st: stomach; sv: horn of sinus venosus.

upstream and confers expression in the thyroid, pharyngeal endoderm, distal stomach, and spleen at E11.5–13.5, with minimal cardiac expression (Reecy et al., 1999; Lien et al., 1999; Schwartz and Olson, 1999).

We cloned a 1956 bp genomic fragment covering bases −1608 to −3563 upstream of the *Nkx2-5* translational start point into a *LacZ* reporter construct. This regulatory region is referred to herein as the *Nkx2-5* Gut Regulatory Sequence (*NGRS*) and the reporter as *NGRS-LacZ*. The *NGRS* contains two evolutionary conserved regions (ECRs; detected using the ECR browser programme (Ovcharenko et al., 2004)), the distal of which shares high sequence identity with the ~300 bp *Xenopus* sequence which drives expression in the gut in transgenic mice (Sparrow et al., 2000). We characterised expression in transgenic mice between E8.5 and E18.5 by examining X-gal staining in whole mounts and sections. Additionally, optical projection tomography (OPT) (Sharpe et al., 2002) of *NGRS-LacZ* embryos permitted three-dimensional analysis of expression in all planes.

NGRS-LacZ gut expression was first detected in a small number of cells in the foregut diverticulum at E8.5 (not shown). Expression was stronger by E9.5, and could be observed in the gut on both sides of the embryo (Figs. 2A, B). The *NGRS* drives expression in the SMP, a structure which is bilateral at E9.5 but grows out to the left by E10.5. *LacZ* expression was localised to the splanchnic mesenchyme and the SMP itself at both stages (Figs. 2E, F), from the level of the posterior stomach down to the pancreatic buds, and was excluded from the gut endoderm. Importantly, the putative splenic mesenchyme, which is located between the SMP and the dorsal pancreatic bud at E10.5, was marked by *LacZ* expression, in agreement with Hecksher-Sorensen et al. (2004).

OPT analysis showed that the bilateral *LacZ* expression at E9.5 is localised to two separate domains, one either side of the midline (Figs. 2C, D). The expression on the left side extends slightly more anteriorly than that on the right (Fig. 2D). By E10.0 the *LacZ* expression has become one continuous domain, with the left and right side expression connected dorsally (data not shown). By E10.5, *LacZ* is expressed in the presumptive pyloric sphincter region and putative splenic mesenchyme (Figs. 2F–H), and has become more left-sided. *LacZ* expression is excluded from the pancreatic endoderm (Fig. 2F).

A single *NGRS-LacZ* gut expression domain still existed at E11.5, corresponding to the pyloric sphincter and putative splenic mesenchyme (Figs. 2I, J). Sections through E11.5 guts confirmed that the pancreatic endoderm within the spleno-pancreatic mesenchyme does not express the transgene. *LacZ*-expressing cells are again observed in the splenic mesenchyme overlying the posterior stomach, but now have a more anterior limit along the greater curvature than at E10.5.

The spleen is recognisable as an elongated structure along the posterior–anterior axis of the stomach by E12.5 (Fig. 2K). The entire spleen is marked by *LacZ* expression at this stage, and this expression remains contiguous with that in the dorsal pancreatic mesenchyme and pyloric sphincter. Sections through the stomach illustrated this connection (Fig. 2L) and demonstrated that, whilst the anterior tip of the spleen appears to be connected to the mesenchyme surrounding the stomach, *NGRS* drives expression only in the splenic component. The E13.5 (Fig. 2M) and E14.5 (Fig. 2N) gut expression patterns were

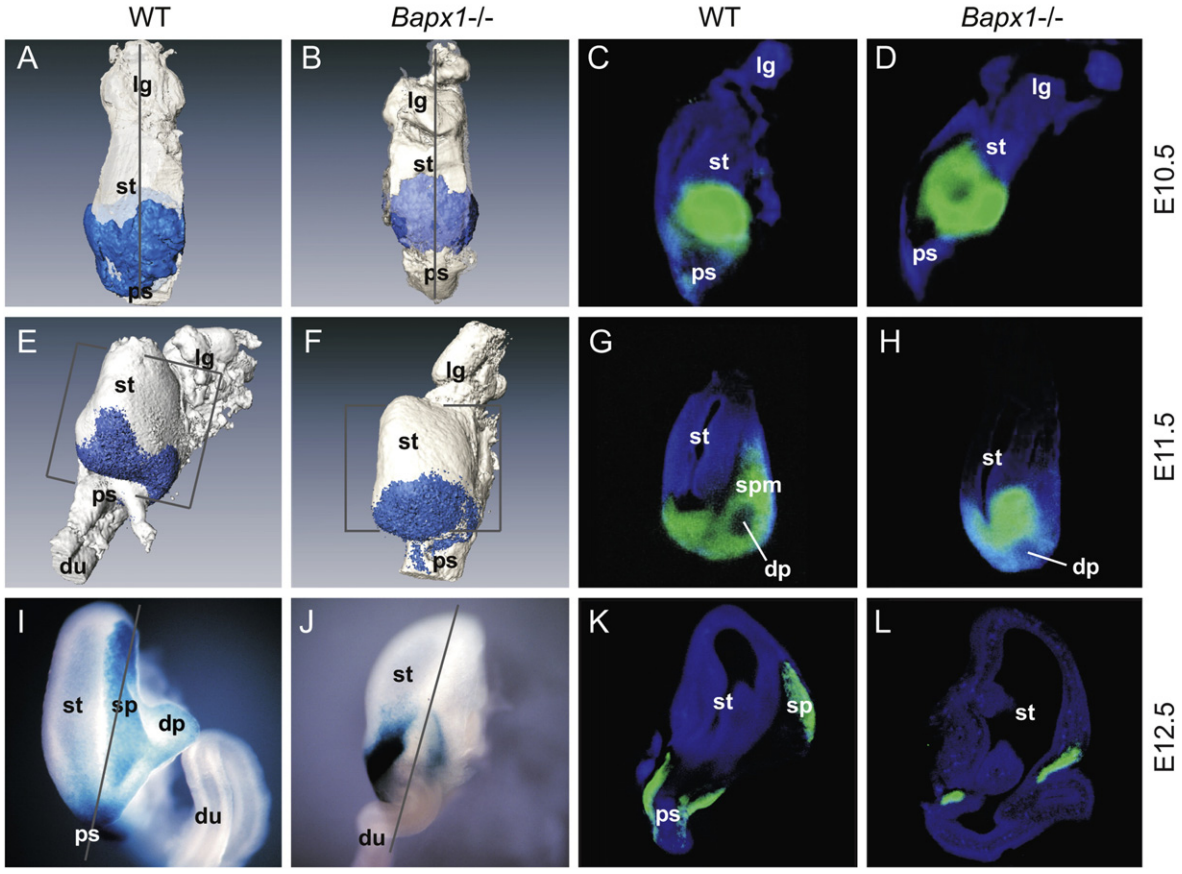

Fig. 3. *NGRS-LacZ* expression is maintained in non-spleen tissues in the absence of Bapx1. *NGRS-LacZ* expression was detected in the posterior stomach and pyloric sphincter of both wild type and *Bapx1*$^{-/-}$ embryos at E10.5 (A–D), E11.5 (E–H), and E12.5 (I–L). Despite the prevalence of expression in the mutant posterior stomach, both the E11.5 splenic mesenchyme (F, H) and E12.5 spleen (J, L) expression domains were absent on the *Bapx1*$^{-/-}$ background. The images in panels A–H, K, L were obtained by OPT, which permitted a more detailed analysis of the staining patterns within the morphologically abnormal *Bapx1*$^{-/-}$ guts. Static images from three-dimensional OPT reconstructions (Amira software) are shown in panels A, B, E, and F. Virtual sections are provided in the third and fourth columns, in which *NGRS-LacZ* expression is shown in green and the anatomy in blue. Sectioning planes are depicted by grey lines. The left horn of the sinus venosus was digitally removed from the gut in panel A to allow visualisation of the signal.

much the same as that established by E12.5, albeit with more intense staining. However, by E16.5 the splenic and pyloric sphincter expression domains have become distinct, the pancreatic mesenchyme expression is lost, and the spleen stains less intensely (Fig. 2O).

Spleen precursors in Bapx1 mutant embryos

Bapx1$^{-/-}$ embryos are asplenic (Lettice et al., 1999). We therefore examined *NGRS-LacZ* expression in *Bapx1*$^{-/-}$ embryos to confirm the identity of the putative splenic precursor cells. *LacZ* expression was maintained in the mutant pyloric sphincter region at E10.5 (Figs. 3A–D), E11.5 (Figs. 3E–H) and E12.5 (Figs. 3I–L), despite the absence of *Bapx1*. Furthermore, expression was observed in the posterior stomach, and around the pancreatic mesenchyme of *Bapx1*$^{-/-}$ embryos, demonstrating that the *NGRS* is not dependent on *Bapx1* for its activity in these tissues. However, both the E11.5 splenic mesenchyme and E12.5 spleen expression domains were absent on the *Bapx1*$^{-/-}$ background (Figs. 3F, J). This is in discordance with the finding made by Brendolan and colleagues that *Nkx2-5* expression is maintained in a splenic structure in the *Bapx1*$^{-/-}$ gut at E12.5 (Brendolan et al., 2005). However, the *Bapx1*$^{-/-}$ mice generated in out laboratory and used in this study (Lettice et al., 1999) do not form such a structure (Asayesh et al., 2006). The twisted, overgrown morphology of the *Bapx1*$^{-/-}$ guts at E12.5 (see for example the disrupted posterior stomach expression domain in Fig. 3J) impeded a detailed examination of the *NGRS-LacZ* staining pattern; however, analysis of virtual OPT sections clearly demonstrated that expression of *NGRS-LacZ* is absent from the region corresponding to the wild type spleen in the *Bapx1*$^{-/-}$ mutants. The loss of *LacZ* expression concomitant with loss of the splenic mesenchyme confirms that the *NGRS* can be used as a marker of spleen cells.

Spleen development in gut culture

The data suggest an initial anterior movement of spleen gene expression from a location posterior to the stomach to an eventual position along the entire left-lateral length of the stomach. To investigate this hypothesis further we developed an organ culture system that would enable manipulation and observation of *NGRS-LacZ*-expressing tissue. Gut tissue was suspended and cultured in Matrigel which appears to adequately support the three-dimensional structure of the stomach and spleen precursors. The gut cultures promoted continual development of the spleen and stomach for up to 48 h in culture. The stomach of the dissected gut (E11.5), at the start of the culture, had an oval shape with a flat anterior stomach (Fig. 4A, arrow) and the oesophagus located at the anterior of the stomach (indicated with oe). After 48 h of culture the gut showed a rounded extended anterior stomach (Fig. 4B, arrow) and the oesophagus was positioned towards the middle of the lesser curvature of the stomach, similar to how the stomach develops *in utero*. This culture system also supports the outgrowth of the dorsal pancreatic bud. Analysis of cell death confirmed that the explants were not undergoing unscheduled cell death in culture (data not shown).

The initial embryonic stage for the cultures (t0) was E11.5. The *NGRS-LacZ* expression pattern in the guts of a number of littermates was examined at the start of each culture assay (n=76). At this stage, *LacZ* expression was located at the posterior of the stomach and around the pyloric sphincter (Fig. 4C). After 48 h in culture, *LacZ* expression had extended anteriorly across the stomach in all cultured explants (n=21). 19% showed partial necrosis of the gut tissue, some staining in the pyloric sphincter area and spleen precursor cells that were not fully condensed in a spleen. However, the majority (81%) showed healthy gut tissue, *LacZ* expression in the pyloric sphincter area, and, crucially, expression in a condensed spleen structure overlying the stomach, extending from the posterior greater curvature towards the anterior lesser curvature of the stomach (Fig. 4D). The extent of spleen development observed across the range of stained t48 guts was greater than that seen in any t0 stained gut.

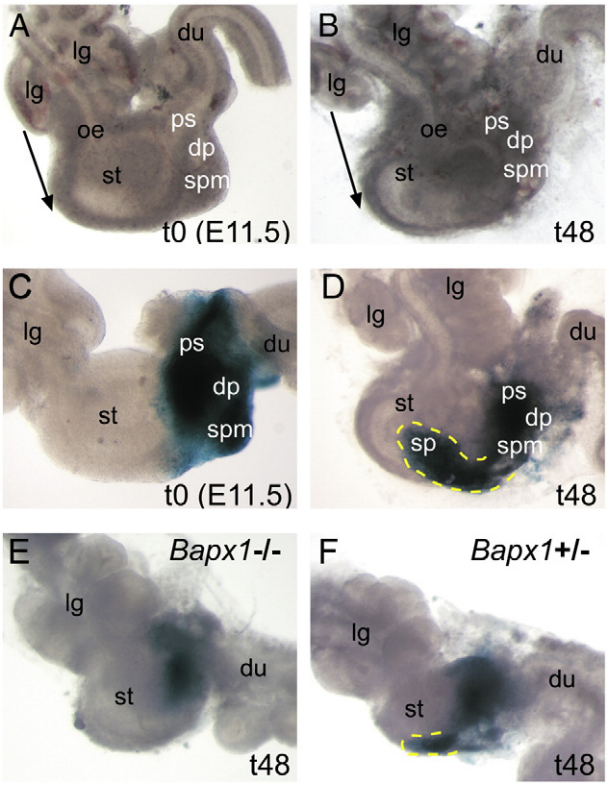

Fig. 4. *NGRS-LacZ* marked spleen development in gut organ culture. A novel Matrigel-based gut culture system was developed in which *NGRS-LacZ* was used as a marker of spleen development. At t0 (A) E11.5 guts have a flat anterior stomach (arrowed), with the oesophagus (oe) lying at the anterior aspect of the stomach. By t48 (B) explants develop an extended rounded anterior stomach (arrowed) and the oesophagus (oe) becomes more posteriorly located, as in *in utero* gut development. (C) Typical X-gal staining for *NGRS-LacZ* expression at t0. In panel D staining is far more extensive by t48 in the area in which the spleen (outlined in yellow) develops—shown here in the explant featured in panels A and B. (E) *Bapx1*$^{-/-}$ guts expressing the *NGRS-LacZ* reporter did not demonstrate spleen formation over the 48 h culture period. (F) Spleen formation (outlined in yellow) was, however, observed in the phenotypically normal heterozygous *Bapx1* mutant gut cultures. Abbreviations: du: duodenum; dp: dorsal pancreatic bud; lg: lung buds; oe: oesophagus; ps: pyloric sphincter; sp: spleen; spm: spleno-pancreatic mesenchyme; st: stomach.

In accordance, cultures of the asplenic *Bapx1*$^{-/-}$ guts at E11.5 (n=9) did not exhibit any spleen formation over the 48 h culture period (Fig. 4E), whereas spleen formation was observed in the heterozygous *Bapx1* mutant gut cultures (n=8) (Fig. 4F), similar to wild type cultures.

Location of NGRS-LacZ-expressing putative spleen precursor cells

We next manipulated the gut culture by removing varying amounts of tissue from the posterior region of the stomach in an attempt to remove putative spleen mesenchyme. This approach was undertaken to confirm that the *LacZ*-expressing cells that were observed in the spleen primordium were derived from the posterior mesenchyme. Removal of posterior mesenchyme proved to have a discernible effect on the *LacZ*-positive cells that populated the splenic region of the stomach. In this series guts from which posterior tissue was removed (n=19) were cultured and all showed a pattern of *LacZ*-positive cells that differed from whole stomach cultures. The observed trends are described below.

Following removal of only the most posterior spleno-pancreatic mesenchyme at the beginning of the culture (Fig. 5A), *LacZ* expression was observed at 48 h in the pyloric sphincter area and in patches within the region the spleen would normally occupy (Fig. 5B).

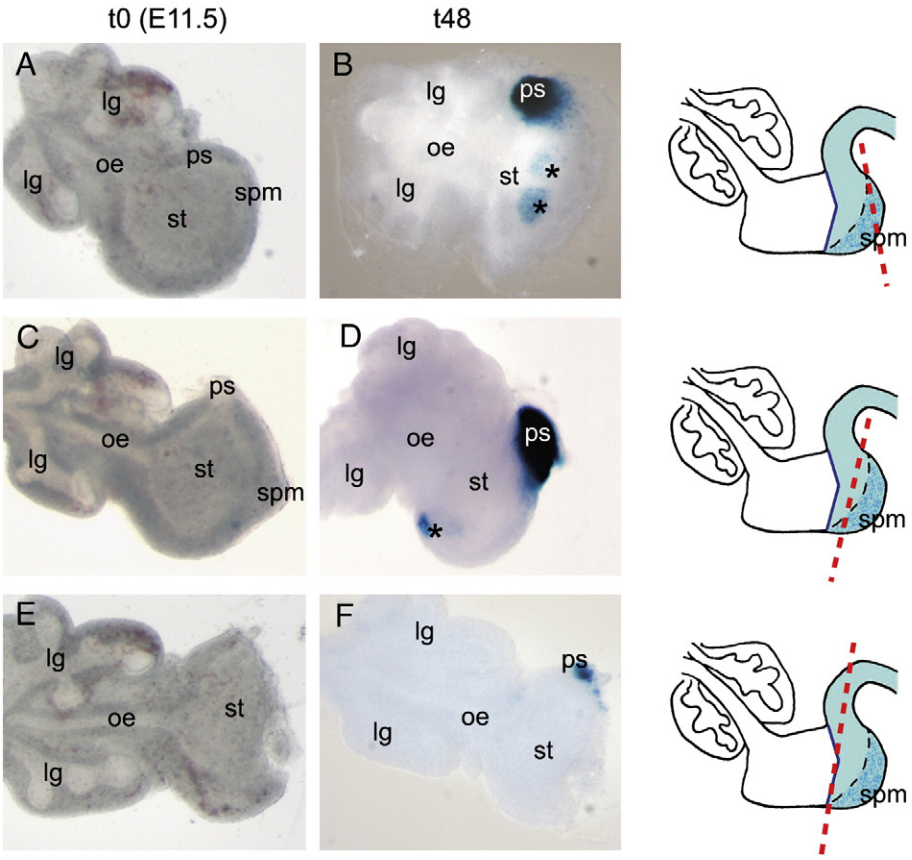

Fig. 5. Location of *NGRS-LacZ*-expressing putative spleen precursor cells. Culture of partial gut explants confirmed the requirement for the putative spleno-pancreatic mesenchyme–but not the stomach–for spleen development, and showed that the *LacZ*-marked spleen tissue expands anteriorly over time. The positions of the cuts used to bisect the guts are represented by red dashed lines on the illustrations to the right of the photographs. The E11.5 (t0) *NGRS-LacZ* expression domain is shaded in blue in these drawings, with the putative spleno-pancreatic mesenchyme shown as mottled blue. (A) Removal of the most posterior spleno-pancreatic mesenchyme at t0 and (B), the same gut at t48, following X-gal staining, showing *LacZ* expression in the pyloric sphincter and in two patches of tissue (asterisked) within the region of normal spleen development. (C) Removal of the posterior stomach and the majority of the putative spleno-pancreatic mesenchyme, with retention of the pyloric sphincter at t0 and (D) the same explant at t48 with expression in the pyloric sphincter and in a small patch of mesenchymal cells (asterisked) at the anterior limit of the stomach. (E) Removal at t0 of all the spleno-pancreatic mesenchyme and the posterior half of the stomach, leaving only a small part of the pyloric sphincter, and (F) the same gut at t48, showing expression in the remaining pyloric sphincter tissue but nowhere else on the explant.

Removal of virtually all the putative spleno-pancreatic mesenchyme and the most posterior part of the stomach, whilst leaving the pyloric sphincter area (Fig. 5C), resulted in expression in the pyloric sphincter area and in a small patch of mesenchymal cells with a faint trail of *LacZ*-positive cells on the anterior part of the stomach (Fig. 5D). Intriguingly, the small patch of marked mesenchymal tissue was located further towards the anterior of the stomach than the intact spleen precursor group in non-manipulated guts (compare Fig. 4D) and than in *in vivo* spleen development (Figs. 2I, J). Following removal of all the spleno-pancreatic mesenchyme and the posterior half of the stomach, leaving only a small part of the pyloric sphincter area (Fig. 5E), *LacZ* expression was detected in the residual pyloric sphincter area but not on the left side of the stomach after 48 h in culture (Fig. 5F). These results suggest that the *LacZ*-expressing tissue is derived from the posterior mesenchyme and expands in an anterior direction over time.

Role of the anterior stomach in spleen development

Since the spleen precursors appear to move towards the anterior tip of the stomach, a possible role for the anterior stomach in spleen development was examined. Gut cultures were performed in which the most anterior part of the stomach was removed ($n=20$). Upon removal (Fig. 6C), the remainder of the stomach would close within 24 h in culture and continue to grow in an anterior direction; however, none of the cultures showed a condensed spleen. Instead, in 100% of the cultures ($n=12$) *LacZ*-expressing cells were observed scattered in the Matrigel, as well as in a small condensation adjacent to the greater curvature of the stomach (Fig. 6D).

To analyse if the putative spleen precursor cells could be redirected towards the lesser curvature of the stomach, we placed the dissected anterior part of the stomach in the lesser curvature ($n=4$) (Fig. 6E). This again resulted in abnormal spleen precursor behaviour. Stained putative spleen precursor cells were found scattered in the Matrigel and adjacent to the posterior greater curvature of the stomach in a partially condensed group (Fig. 6F). However, placing the anterior stomach in the lesser curvature did not result in redirection of a condensed spleen towards this region suggesting that continuity of the tissue is crucial.

Stomach mesenchyme and spleen develop from distinct tissues

The data thus far show that the earliest detectable spleen precursors derive posterior to the stomach and appear to track anteriorly along the left mesenchyme of the stomach. To show that the spleen is indeed derived from the posterior rudiment and thus there is little or no contribution from the neighbouring stomach mesenchyme, we used a second marking method (Arques et al., 2007) that enables retrospective examination of marked clones. This approach was initially used to study cell lineage in the limb bud. The mouse line *RERT* carries a conditional Cre recombinase (*IRES-CreERT2*) which is targeted to the ubiquitously expressed *Pol2* gene (Guerra et al., 2003). The Cre activity is inducible by

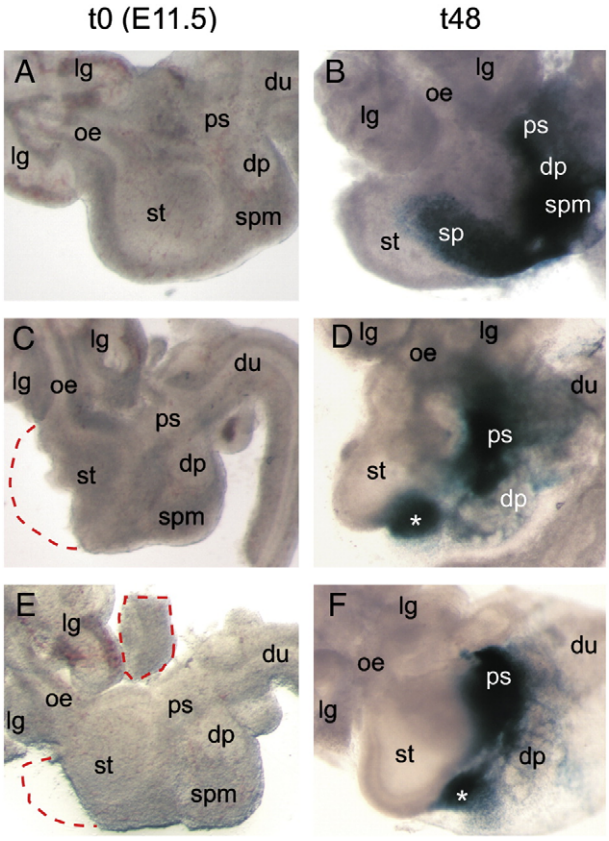

Fig. 6. The anterior stomach plays a role in directed spleen development. The distribution of *LacZ*-expressing putative spleen precursors was examined in the presence and absence of the most anterior part of the stomach. (A, B) *LacZ*-positive cells were arranged in a condensed structure overlying the stomach in the majority (81%) of intact explants. However, when the anterior part of the stomach was removed (dashed red line) as in panel C, none of the cultures developed a condensed spleen by t48 (D); the *LacZ*-expressing cells instead became scattered in the Matrigel, as well as in a small condensation adjacent to the greater curvature of the stomach (asterisk in panel D). In panel E, the dissected anterior stomach (bounded by dashed red line) was placed in the lesser curvature of the stomach to analyse whether the putative spleen precursor cells could be redirected towards this tissue, but once again *LacZ*-positive cells (F) were found scattered in the Matrigel and in a partially condensed group (asterisked) adjacent to the posterior greater curvature. Redirection towards the ectopically placed anterior stomach region did not occur. Abbreviations: dp: dorsal pancreatic bud.

the administration of tamoxifen to the pregnant female. Crossing of the *RERT* line to mice carrying the *R26R* reporter (Soriano, 1999) gene generates embryos with tamoxifen-inducible *LacZ* expression. The location and distribution of groups of daughter cells provide information on shared or separate precursor groups.

A single injection of tamoxifen generates *LacZ*-positive cells observable after 6 h and most recombination events are estimated to occur between 12 and 18 h with little increase in clone frequency after 24 h (Arques et al., 2007). Here, *LacZ*-expressing cells were induced on the seventh (E7.7) and eighth days (E8.5-8.7) of development at stages sufficiently early to enable Cre-mediated recombination to occur before the appearance of the spleen. Embryos were sacrificed at E11.5–E14.5 and assayed for *LacZ* expression. 332 embryos labelled for *LacZ* expression were analysed in this study. The positive cells were observed in distinct groups randomly distributed within the embryos (personal communication and Arques et al., 2007). From the 332 *LacZ*-positive embryos, 41 displayed clones in the stomach, spleen, and/or intestines.

We focused on the marked cells within the stomach, spleen and pancreas. Tamoxifen administered at E7.7 generated *LacZ*-positive cells in spleen tissue ($n=3$) (Table 1; a more extensive presentation of the data is provided in Table S1) but importantly, no detectable staining in the adjacent stomach (Figs. 7C, D). Conversely, a subset of embryos ($n=8$) showed *LacZ* expression in the left-side stomach mesenchyme but no staining in the spleen. Tamoxifen treatment at E8.5–E8.7 produced 24 embryos (Table 1) that were *LacZ*-positive and of these 15 labelled only in the stomach. Staining in the stomach was localised to the epithelium ($n=1$) or in the mesenchyme on the right side only ($n=3$), left side only ($n=4$) or on both sides ($n=7$). No staining was found in the splenic mesenchyme of these 24 guts, despite the extensive marking in many of them (suggestive of recombinase-mediated conversion of multiple clones) (Figs. 7A, B). The remaining five embryos at E7.7 and nine embryos at E8.5–8.7 stained in both the stomach and the splenic mesenchyme. Multiple independent clones were induced in these embryos most likely accounting for the extensive staining. These data suggest that there is little or no mixing between the spleen and the adjacent stomach mesenchyme and that the stomach and spleen mesenchymal cells are separately inducible populations.

The model that the left-side stomach mesenchyme does not contribute to the spleen is supported by these data. We therefore surmise that the spleen originates posterior to the stomach and, as it develops, moves along the left lateral aspect of the stomach to adopt its appropriate position.

Discussion

In their 1921 paper on the development of the mammalian spleen, Thiel and Downey stated: "To anyone looking over the literature on the development of the mammalian spleen the necessity for more

Table 1
Location of marked cells in clonal analysis assay

Time injected	Time harvested	LacZ+ve mice	Informative guts[a]	Stomach AND spleen	Stomach only[b]					Spleen only[c]
					Epi	R	L	RL	Total	
E7.7	E11.7	12	9	3				4	4	2
	E12.5–12.7	28	7	2			2	2	4	1
	E13.5	37	0							
	E14.5	6	1		1				1	
E8.5–8.7	E12.5–12.7	73	14	5	1	2	4	2	9	
	E13.5–13.7	97	10	4		1		5	6	
	E14.5	13	0							
Total		266	41	14	2	3	6	13	24	3

The table provides details of the numbers of guts exhibiting marked clones in each tissue type. Embryos received tamoxifen via maternal injection and were harvested at the embryonic (E) days listed in the table. Only embryos exhibiting X-gal staining ("LacZ+mice") are included in this table.

[a]"Informative guts" are those showing strong staining ascribable to specific tissues, with no general staining and no damage; guts not meeting these criteria are excluded from this table (details can, however, be found in Table S1). The number of guts in which specific LacZ expression domains were noted is listed under "Stomach AND spleen", "Stomach only", and "Spleen only".

[b]Stomach-only staining is further subdivided into epithelium only (epi), right-side mesenchyme (R), left-side mesenchyme (L), and that found in mesenchyme on both sides of the stomach (RL); guts classed as showing mesenchymal staining may also show staining in the stomach epithelium.

[c]The final category represents guts with either staining in the spleen only or in the spleen with some marking of the right-sided mesenchyme.

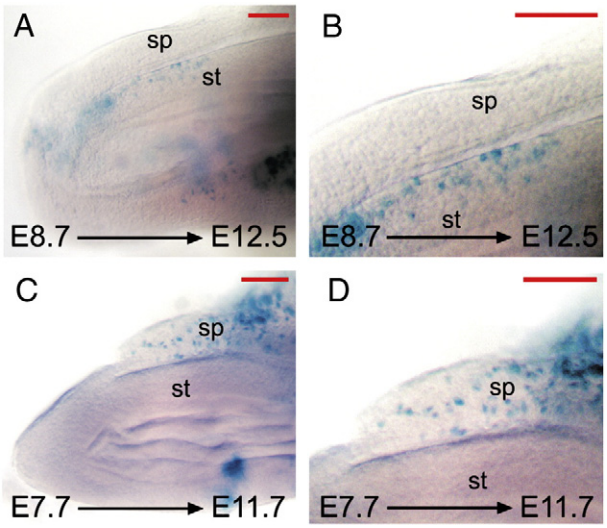

Fig. 7. Clonal analysis reveals separate origins of the stomach and spleen mesenchyme. Tamoxifen was administered *in utero* to embryos resulting in induction of *LacZ* expression in a limited number of cells and their descendents. Retrospective analysis of the distribution of the marked clones revealed a number of patterns, most crucially the marking of cells in the mesenchyme of the stomach (st) but not of the adjacent spleen (sp) [(A), high magnification view in (B)], and the marking of cells in the spleen but not the adjacent stomach mesenchyme [(C), high magnification view in (D)]. These distributions are indicative of separate origins of the stomach and spleen mesenchyme. Images are of whole mount guts. The injection and culling times, in embryonic days (E), are written along the bottom of each image. The tamoxifen dosages used, in μg/g maternal weight, were: (A, B) 2.5 μg/g, (C, D) 15 μg/g. Red scale bars represent 100 μm. oe: oesophagus; sp: spleen; st: stomach.

work on the finer details of the process is evident". This statement has held true for the initial events in spleen morphogenesis until fairly recently, with the discovery of a number of markers and mutants of early spleen development (Hecksher-Sorensen et al., 2004; Brendolan et al., 2007). However, many questions remain about the origins and early morphogenetic processes of the spleen.

We took a two-pronged approach to focus on the early events in spleen morphogenesis. The first was to analyse gut morphogenesis in organ culture. This approach relied on a novel spleen reporter gene. The *NGRS* was cloned on the basis of previous reports of a regulatory element in this region that confers spleen expression (Reecy et al., 1999). Expression analysis of an *NGRS-LacZ* reporter gene revealed that the *NGRS* can drive expression from very early stages of spleen development and so proved valuable in examining the dynamics of spleen morphogenesis in culture.

The second approach analysed clonally derived cells and their descendents. The condensation of mesenchyme that is first identifiable as spleen is found in the dorsal mesogastrium adjacent to the stomach. The cell population that gives rise to the spleen is not derived from the stomach mesenchyme. These data are consistent with our previous suggestions which cite mesenchyme posterior to the stomach and associated with the pancreas (we termed this spleno-pancreatic mesenchyme) as the origin of spleen cells (Hecksher-Sorensen et al., 2004).

The data from our culture experiments are consistent with a posterior origin of the spleen. In addition the spleen precursor cells marked by *LacZ* expression expand from the spleno-pancreatic mesenchyme through the mesenchyme overlying the left side of the stomach towards the anterior lesser curvature of the stomach in a diagonal direction. A signal from the anterior part of the stomach appears to drive this outgrowth, as removal of the anterior stomach resulted in disorganised spleen tissue expansion in all of the cultures. The spleen precursor cells consequently failed to move from the posterior greater curvature of the stomach towards the anterior lesser curvature. Removal of the anterior tip of the stomach may therefore result in elimination of a chemo-attractant signal. Alternatively, the anterior stomach may be required to physically direct the cells into position or to act as a scaffold for this organisation.

The spleno-pancreatic mesenchyme underlies the SMP at E10.5 and expresses a number of spleen marker genes, including *Nkx2-5* (Hecksher-Sorensen et al., 2004). Splenic expression is already located on the left side of the embryo at this stage. *XNkx2-5* is also a marker of left-sided spleen tissue in *Xenopus*; however, expression can be detected

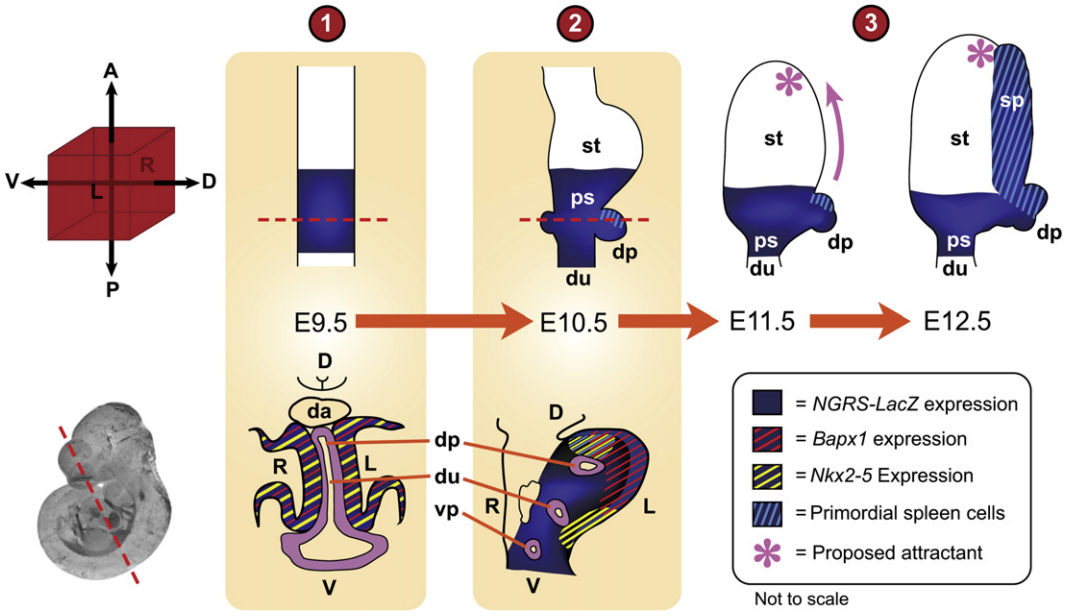

Fig. 8. A model for spleen morphogenesis. 1: Markers of the wholly left-sided spleen (*Bapx1*, *Nkx2-5*, *NGRS*) are initially expressed bilaterally (left and right side) in the SMP, similar to *Xenopus*. 2: Spleen cells derive from mesenchyme posterior to and distinct from the stomach. Light blue hatching denotes these primordial spleen cells. 3: *NGRS-LacZ*$^{+}$ spleen cells (possibly within a leading edge) then move from the posterior mesenchyme and track along the dorsal face of the stomach towards an anterior attractant. The top row of figures shows guts at progressive stages of development, viewed from the left side (later the greater curvature). The bottom row contains cross-sections, taken at the levels indicated by dashed red lines, through the E9.5 and E10.5 SMP. Abbreviations: A: anterior; D: dorsal; da: dorsal aorta; dp: dorsal pancreas; du: duodenum; L: left; P: posterior; ps: pyloric sphincter; R: right; sp: spleen; st: stomach; V: ventral.

prior to this in two pools of spleen precursor cells, one on either side in the embryo (Patterson et al., 2000). In this study we showed that an upstream regulatory region of the spleen marker gene *Nkx2-5* (the *NGRS*) can be used to label spleen precursors from the very earliest stages of spleen development. The marking of cells on both sides of the SMP at E9.5 by both *Nkx2-5* and *NGRS-LacZ* expression, 24 h before the earliest previously described spleen markers, suggests that spleen precursors initially exist bilaterally. *Bapx1* is also expressed at this stage in an overlapping pattern, supporting a bilateral origin of the spleen (Hecksher-Sorensen et al., 2004). The similarity of these expression patterns to the originally bilateral distribution of *XNkx2-5*-expressing spleen precursor cells in *Xenopus* also supports this notion (Patterson et al., 2000). An initially bilateral population of spleen precursors could be invoked as an explanation for polysplenia and right-sided spleen associated with heterotaxy.

Removal of differing portions of the spleno-pancreatic mesenchyme demonstrated that the spleen precursor cells originate from the spleno-pancreatic mesenchyme and expand through the mesenchyme overlying the left side of the stomach in an anterior direction. Interestingly, if a small part of spleno-pancreatic mesenchyme was left attached to the posterior stomach at the beginning of the culture period, the spleen precursor cells therein seemed to have an increased migratory response towards the proposed anterior signal (Fig. 5D). This may suggest that not all spleen precursor cells have the same capacity to respond to signals and thus should not be seen as a homogenous group. Cells undergoing organogenesis are rarely found as migrating chemotactic individuals, but usually migrate as a collective; however, differences in migratory response between cells within a cohesive group have been described (Haas and Gilmour, 2006). Extrinsic cues often instruct a small number of peripheral leading edge cells which then instruct follower cells, instead of acting on all members of a cohort (Lecaudey and Gilmour, 2006). The leading edge theory may provide a mechanism for spleen morphogenesis. The next stage in dissecting the dynamics of spleen morphogenesis will be to tag cells in different regions of the spleen primordium and examine their contribution using live cell imaging.

The findings presented in this paper are summarised in Fig. 8. Our studies provided a number of insights, including: the location of spleen precursor cells in the spleno-pancreatic mesenchyme alongside the posterior stomach, the requirement for the anterior stomach for the correct anterior extension of spleen development (possibly by migration), and the possibility of differential cell roles within the developing spleen. Loss of splenotypic *NGRS-LacZ* expression in *Bapx1*$^{-/-}$ embryos confirmed that spleen development was indeed being observed in our studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.03.031.

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