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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.

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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 (Meibius 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 side of the body is abnormal, resulting in cardiovascular abnormalities and a range of spleen defects; these include asplenia, polysplenia (spleen tissue on both sides of the body), 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 (Thiell 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, Tbx1 (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; Raclely 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...
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<sup>+/−</sup> 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: ACTTGAACACCCGCGCGAGGCT, R: TTAATAGCCTCACTAGGCGTGCG ATCCCGTAAGTTC). 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, Pernis, Cramlington, UK) and 1% penicillin/streptomycin (Sigma, Dorset, UK), from NGRS-LacZ, Bapx1<sup>+/−</sup> NGRS-LacZ, or Bapx1<sup>+/−</sup> 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 in 5% CO₂; subsequently 200 µl supplemented DMEM was added and the cultures grown for 48 h at 37°C and 5% CO₂. 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 the R26R reporter (floxed lacZ inserted in the ROSA26 locus) (Soriano, 1999) and RERT (JRES-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-Sorensen 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-Sorensen et al., 2004). Abbreviations: D: dorsal; dp: dorsal pancreatic bud; du: duodenum; f: forelimb; L: left; nt: neural tube; R: right; V: ventral; vp: ventral pancreatic bud.](image-url)
**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.
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 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

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**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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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. 20).

**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 our 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) impedes 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 is 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 anterior stomach (Fig. 4B, arrow) and the oesophagus was examined at the start of each culture assay (Fig. 4C, oe). After 48 h in culture, had an oval shape with a posterior greater curvature 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 48 h guts was greater than that seen in any t0 stained gut.

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).
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
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–E8.7 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

![Image](https://example.com/image.png)

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 (81%) of intact explants. 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 6 days in culture (D); the (dashed red line) as in panel C, none of the cultures developed a condensed spleen by 6 days in culture (D). In panel E, the dissected anterior stomach (bounded by dashed red line) was placed to the posterior greater curvature. Redirection towards the ectopically placed anterior stomach mesenchyme and that the stomach and spleen develops, moves along the left lateral aspect of the stomach to adopt its appropriate position.

Table 1

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<th>Informative guts</th>
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<th>Spleen only&lt;sup&gt;c&lt;/sup&gt;</th>
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<td></td>
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<td>97</td>
<td>10</td>
<td>4</td>
<td>1 5 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E14.5</td>
<td>13</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>266</td>
<td>41</td>
<td>14</td>
<td>2 3 6 13 24 3</td>
<td></td>
</tr>
</tbody>
</table>

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.

<sup>a</sup>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”.

<sup>b</sup>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.

<sup>c</sup>The final category represents guts with either staining in the spleen only or in the spleen with some marking of the right-sided mesenchyme.
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...
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 Xnkl2k-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 between cells within a cohesive group have been described usually migrate as a collective; however, differences in migratory response of spleen precursor cells to the anterior stomach at the beginning of the culture period, the spleen precursor cells therein seemed to have an increased 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 along 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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