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Localised inhibition of FGF signalling in the third pharyngeal pouch is required for normal thymus and parathyroid organogenesis

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SUMMARY

The thymus and parathyroid glands are derived from the third pharyngeal pouch endoderm. The mechanisms that establish distinct molecular domains in the third pouch and control the subsequent separation of these organ primordia from the pharynx are poorly understood. Here, we report that mouse embryos that lack two FGF feedback antagonists, Spry1 and Spry2, display parathyroid and thymus hypoplasia and a failure of these organ primordia to completely separate from the pharynx. We show that FGF ligands and downstream reporter genes are expressed in highly regionalised patterns in the third pouch and that sprouty gene deletion results in upregulated FGF signalling throughout the pouch endoderm. As a consequence, the initiation of markers of parathyroid and thymus fate is altered. In addition, a normal apoptotic programme that is associated with the separation of the primordia from the pharynx is disrupted, resulting in the maintenance of a thymus-pharynx attachment and a subsequent inability of the thymus to migrate to its appropriate position above the heart. We demonstrate that the sprouty genes function in the pharyngeal endoderm itself to control these processes and that the defects in sprouty-deficient mutants are, at least in part, due to hyper-responsiveness to Fgf8. Finally, we provide evidence to suggest that parathyroid hypoplasia in these mutants is due to early gene expression defects in the third pouch, whereas thymus hypoplasia is caused by reduced proliferation of thymic epithelial cells in the thymus primordium.

KEY WORDS: FGF, Sprouty, Thymus, Parathyroid, Pharyngeal pouch, Endoderm, Mouse

INTRODUCTION

The thymus is a bi-lobed organ that is located just above the heart and is the site of thymocyte selection and T-cell differentiation. The parathyroid glands are located next to the thyroid gland and regulate calcium homeostasis through the production of parathyroid hormone. Despite their unrelated functions, the thymus and parathyroid share a common developmental origin in that they are both derived from the third pharyngeal pouch (reviewed by Blackburn and Manley, 2004; Gordon and Manley, 2011). Four pairs of pharyngeal pouches arise sequentially in the mouse embryo from anterior to posterior by the bilateral outgrowth of the pharyngeal endoderm. As the evaginating pouches make contact with the pharyngeal ectoderm, the pharyngeal region is segmented into pharyngeal arches (Graham, 2003; Cordier and Haumont, 1980).

After pouch formation, the third pouch becomes subdivided into two molecularly distinct domains that can be visualised by embryonic day (E) 10.5 of mouse development (Gordon et al., 2001). An anterior-dorsal domain, which is fated to differentiate into the parathyroid, can be distinguished by the expression of glial cells missing 2 (*Gcm2*), which is required for parathyroid differentiation and survival (Günther et al., 2000; Liu et al., 2007). A prospective thymus domain is marked by *Bmp4* expression at the posterior-ventral side of the pouch at E10.5 (Patel et al., 2006). The expression of the first definitive thymus marker, *Foxn1*, appears in the posterior, *Bmp4*-positive pouch by E11.25. *Foxn1* expression spreads further along the posterior side of the pouch towards the *Gcm2*+ region and this *Foxn1*+ domain will form the primordium of the thymus (Gordon et al., 2001). After the establishment of two molecularly distinct organ primordia, they physically separate from each other and from the pharynx and migrate to positions at the mediastinum anterior-ventral to the heart (thymus) and adjacent to the thyroid (parathyroid). Little is known about the mechanisms that allow the separation of organs from the pharynx. Apoptotic epithelial cells have been detected in the region where the thymus separates from the pharynx at E11.75 just prior to separation, suggesting that apoptosis might be required for the separation process (Gordon et al., 2004). Although *Pax1*, *Pax9* and *Hoxa3* have been shown to be necessary for the normal separation of the thymus primordium from the pharynx, these studies did not investigate whether the lack of separation is associated with the loss of apoptosis in these mutants (Su et al., 2001; Hetzer-Egger et al., 2002).

Our understanding of the identity and actions of signalling molecules that coordinate pouch formation and subsequent steps in thymus and parathyroid organogenesis is still incomplete. The signalling cascades that control *Gcm2* and *Foxn1* expression are complex and several pathways and transcription factors have been implicated. These include a Hox-Eya-Six-Pax gene network required for initiation and maintenance of organ-specific gene expression (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995; Su et al., 2001; Zou et al., 2006). Wnt signalling, presumably as a result of *Wnt4* and *Wnt5b* expression in the pharyngeal region, has been implicated as a regulator of *Foxn1* expression (Balciunaite et al., 2002). The transcription factor Gata3, which is mutated in HDR (hypoparathyroidism, sensorineural deafness and renal

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disease) syndrome, directly regulates the expression of human homologue of *Gcm2*, *GCMB* (*GCM2* – Human Gene Nomenclature Database) (Grigorieva et al., 2010). In addition, the bone morphogenetic protein (BMP) and sonic hedgehog (Shh) signalling pathways are required for normal thymus and parathyroid organogenesis, respectively (Ohnemus et al., 2002; Storm et al., 2003; Bleul and Boehm, 2005; Moore-Scott and Manley, 2005; Soza-Ried et al., 2008; Gordon et al., 2010; Neves et al., 2012).

Fibroblast growth factor (FGF) signalling regulates the development of several tissues in the pharyngeal region. Mutations that affect FGF signalling have been shown to affect two developmental processes that impact on thymus and/or parathyroid organogenesis: (1) *Fgf8* hypomorphic mutants fail to form normal third pouches resulting in thymus and parathyroid aplasia or hypoplasia (Abu-Issa et al., 2002; Frank et al., 2002); and (2) thymic epithelial cell (TEC) proliferation after E12.5 is reduced in Fgfr2(IIIb) and Fgf10 mutants (Ohuchi et al., 2000; Revest et al., 2001). Thus, although FGF signalling has been implicated in third pouch formation (~E9.25) and TEC proliferation (after E12.5), the role of this pathway between E10.5 and E12.5 has not been investigated directly. Research over the last few years has shown that FGF feedback antagonists of the sprouty gene family are important regulators of organogenesis (Mason et al., 2006). In this study, we report that the localised inhibition of FGF signalling by sprouty proteins is essential for several key processes during thymus/parathyroid organogenesis: (1) the normal initiation of *Gcm2*, *Bmp4* and *Foxn1* expression in the third pouch, (2) apoptosis in the third pouch that is associated with organ detachment from the pharynx, and (3) Fgf10-mediated thymus growth after E12.5.

MATERIALS AND METHODS

Generation of embryos

Mutant mouse lines were maintained on a mixed (129SvEv,C57BL/6J,FVB/N) genetic background, and genotyped by PCR as described in original publications. Mutant lines harbouring conditional (floxed) *Spry1* and *Spry2* alleles have been described (Basson et al., 2005; Shim et al., 2005). These mice were crossed to *βactinCre* mice to generate *Spry1* and *Spry2* null alleles (Lewandoski et al., 1997). Embryos lacking both genes (*Spry1;2dko*) were produced by crossing *βactinCre/βactinCre;Spry1*$^{+/-}$*;Spry2*$^{+/-}$ males with *Spry1*$^{flox/flox}$*;Spry2*$^{flox/flox}$ females, as described (Simrick et al., 2011). For rescue experiments, *βactinCre*^{2}*;Spry1*$^{+/-}$*;Spry2*$^{+/-}$*;Fgf8*$^{+/-}$ males were crossed with *Spry1*$^{flox/flox}$*;Spry2*$^{flox/flox}$ females or *βactinCre*^{2}*;Spry1*$^{+/-}$*;Spry2*$^{+/-}$ males with *Spry1*$^{flox/flox}$*;Spry2*$^{flox/flox}$*;Fgf3*$^{+/-}$ females. The *Fgf3* and *Fgf8* null alleles have been described (Meyers et al., 1998; Alvarez et al., 2003). Endoderm-specific mutants were produced using *Sox17-2A-iCre* (Engert, S. et al., 2009) by crossing *Sox17-2A-iCre;Spry1*$^{flox/+}$*;Spry2*$^{flox/+}$ males to *Spry1*$^{flox/flox}$*;Spry2*$^{flox/flox}$ females.

Noon on the day a vaginal plug was detected was designated as E0.5. Embryos were accurately staged by determining the number of somite pairs, indicated as somite stage (ss). All experiments involving mice were approved by the UK Home Office. At least three separate embryos of each genotype were analysed per experiment.

Histology, in situ hybridisation and immunohistochemistry

Embryos were dissected in PBS and fixed overnight in 4% paraformaldehyde in PBS, followed by dehydration through a series of ethanol washes and embedding in wax. Sections were cut at 7 μm and dried overnight at 42°C. In situ hybridisation (ISH) was performed according to standard methods (Yaguchi et al., 2009). The probes used for section ISH have all been previously described as follows: *Fgf3* (Robinson et al., 1998), *Fgf8* (Crossley and Martin, 1995), *Fgf15* (Borello et al., 2008), *Etv4* and *Etv5* (Klein et al., 2006), *Dusp6* (Dickinson et al., 2002), *Foxn1* and *Gcm2* (Gordon et al., 2001), *Spry1* and *Spry2* (Minowada et al., 1999). Immunohistochemistry (IHC) was performed according to standard methods (Yu et al., 2009). Double IHC was performed using primary antibodies raised in rabbit against activated caspase-6 (Abcam #ab52295) to analyse apoptosis, and raised in mouse against E-cadherin (Fitzgerald #02660) to label epithelial cell membranes. A rabbit antibody to phospho-histone H3 (Ser10) (Cell Signaling #9701) was used to detect proliferating cells. Secondary Alexa fluorophore-labelled antibodies were obtained from Invitrogen. All primary and secondary antibodies were diluted at 1/200. Images were acquired with a 5M pixel Nikon DS colour camera connected to a Nikon Eclipse 80i microscope and subsequently collated and annotated using Adobe Photoshop and Illustrator.

Three-dimensional reconstruction of the pharyngeal apparatus

Embryos for three-dimensional (3D) reconstruction of the pharyngeal region were sectioned in the coronal plane, stained with Haematoxylin and Eosin (H&E) and photographed as detailed above. 3D reconstruction from alternate sections was carried out using Winsurf version 4.3, as described by Patel et al. (Patel et al., 2006). Relative volumes of each organ/structure were determined. The mean volume of control glands was calculated, and the volumes of littermate controls and knockouts calculated as a percentage of this mean control volume. When no stage-matched littermate controls were available, knockouts were compared with the mean volume of all stage-matched *Spry1*$^{flox/+}$*;Spry2*$^{flox/+}$ controls collected during the course of the study. Statistical analyses were carried out using Graphpad and Microsoft Excel.

RESULTS

FGF signalling is highly regionalised in the developing third pharyngeal pouch

At E10.5 of development in the mouse, distinct, molecularly defined domains can be observed in the third pharyngeal pouch. An anterior-dorsal, *Gcm2*-positive domain represents the primordium of the parathyroid (Fig. 1A,K). At this stage of development, the ventral pouch can be identified by *Bmp4* expression (Fig. 1B,K). Cells in this ventral *Bmp4*+ domain turn on *Foxn1* expression from E11 and *Foxn1*+ cells, destined to become the thymus, occupy a ventral-posterior region of the third pouch, complementary to the *Gcm2*+ region, by E11.5 (Gordon et al., 2001). As a first step towards understanding whether FGF signalling plays a role in thymus/parathyroid development between E10.5 and E11.5, we determined the expression of genes encoding FGF ligands and downstream targets in and around the third pouch at E10.5. We focused this analysis on FGF genes that had been implicated in pharyngeal or thymus development in previous studies (Revest et al., 2001; Abu-Issa et al., 2002; Frank et al., 2002; Trokovic et al., 2005; Aggarwal et al., 2006). In situ hybridisation studies on sagittal sections through the third pouch identified a small region in the posterior pouch that expresses *Fgf3*, *Fgf8* and *Fgf15* (Fig. 1C-E). *Fgf10* is expressed in the neural crest-derived mesenchyme surrounding the pouch, and in a small domain in the most ventral part of the *Bmp4*+ pouch region (Fig. 1F). To identify regions of active FGF signalling in the pouch, we determined the expression of *Spry1* and *Spry2*, as these genes are induced by FGF signalling in several developmental contexts (Minowada et al., 1999). *Spry1* and *Spry2* are expressed at low levels throughout most of the pouch, with highest levels of expression in and around the *Fgf3/8/15*-expressing region in the posterior pouch (Fig. 1G,H). These two sprouty genes are also expressed in the neural crest surrounding the pouch, with highest expression in the cells adjacent to the *Fgf3/8/15/Spry* expression domain in the posterior pouch (Fig. 1G,H). Taken together, these

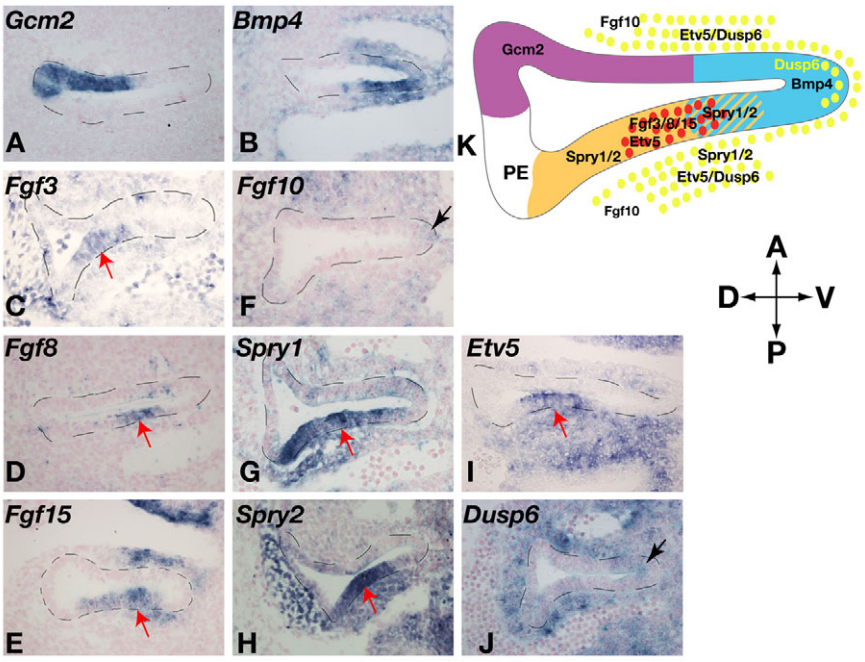

Fig. 1. FGF gene expression in the third pharyngeal pouch at E10.5. (**A-J**) Gene expression patterns in sagittal sections through the third pharyngeal pouch of wild-type E10.5 mouse embryos, with the anterior side of the pouch oriented to the top and the dorsal side to the left in all panels. The pharyngeal pouch is outlined by dashed lines to aid visualisation. The red arrow indicates a region of FGF-expressing cells in the posterior pouch (C-E,G-I), with high sprouty expression (G,H) and low levels of *Etv5* expression (I). Black arrows indicate low *Fgf10* (F) and *Dusp6* (J) expression in the ventral pouch. (**K**) Gene expression patterns summarised in a cartoon of the third pharyngeal pouch as seen in sagittal sections. The dorsal-anterior *Gcm2*+ domain that will form the parathyroid and the ventral *Bmp4*+ presumptive thymus domain are indicated in purple and blue, respectively. *Fgf3/8/15*-expressing cells in the posterior pouch are represented by red circles and the region of the pouch endoderm that expresses high amounts of *Spry1/2*, is indicated in yellow. FGF-responsive cells in the surrounding neural crest or ventral pouch as marked by *Etv4/5*, *Dusp6* and/or *Spry1/2* expression are represented by yellow circles. PE indicates the dorsal pharyngeal endoderm that lines the pharynx. A, anterior; D, dorsal; P, posterior; V, ventral.

observations suggest that FGF ligands, produced in the posterior pouch endoderm and the adjacent neural crest, can induce *Spry1* and *Spry2* expression in both tissues.

Next, we visualised the expression of three additional transcriptional targets of the FGF pathway: *Etv4* (*Pea3*), *Etv5* (*Erm*) (Roehl and Nüsslein-Volhard, 2001; Klein et al., 2008) and *Dusp6* (*Mkp3*) (Li et al., 2007). High *Etv4* (Fig. 2A) and *Etv5* expression was evident in the surrounding neural crest, especially along the anterior and posterior sides of the pouch, confirming that these cells are responding to FGF signals (Fig. 1I). By contrast, the pouch endoderm appeared to be largely devoid of *Etv4* and *Etv5* expression, with the exception of a distinct expression domain in the posterior pouch where FGF ligands are expressed (Fig. 1I). *Dusp6* is also expressed at high levels in the neural crest, with low expression in a small region in the ventral pouch where *Bmp4* and *Fgf10* are expressed, but no detectable expression in the posterior pouch (Fig. 1J). The observation that several genes that report FGF signalling are expressed in and around the third pouch suggests that FGF signalling is active during pouch patterning. Although these genes show slightly different expression patterns, which probably reflects subtle differences in gene regulatory mechanisms, our data suggest the presence of an FGF signalling centre in the posterior pouch endoderm and a potential negative feedback mechanism whereby sprouty inhibits intracellular FGF signal transduction in the pouch endoderm.

By comparing the expression patterns of FGF pathway genes with other region-specific markers, we found that the FGF signalling centre was localised in the posterior pouch between the anterior-dorsal *Gcm2*+ parathyroid domain and the ventral *Bmp4*+ thymus domain, with some overlap with the latter (summarised in Fig. 1K). Taken together, these observations suggest that strong negative feedback mechanisms have evolved to inhibit the activation of FGF signalling in the third pharyngeal pouch and that *Spry1* and *Spry2* might serve essential functions during third pouch patterning and subsequent events that affect thymus/parathyroid development.

Sprouty restricts FGF signalling to specific regions in and around the third pouch

To test the hypothesis that Spry1 and Spry2 regulate thymus/parathyroid development by preventing excessive FGF signalling in the pouch endoderm, we generated *Spry1*$^{-/-}$;*Spry2*$^{-/-}$ (*Spry1;2dko*) embryos. *Spry1*$^{+/-}$;*Spry2*$^{+/-}$ (*Spry1;2*$^{+/-}$) littermate embryos produced in these crosses were normal compared with wild-type embryos with respect to thymus and parathyroid development and were used as controls. We first compared the expression of three FGF reporter genes, *Etv4*, *Etv5* and *Dusp6*, in *Spry1;2*$^{+/-}$ control and *Spry1;2dko* embryos (Fig. 2A-F). As shown in Fig. 2, the regionalised *Etv4*, *Etv5* and *Dusp6* expression patterns were almost entirely disrupted in the *Spry1;2dko* embryos and these genes were ubiquitously expressed at high levels throughout the pouch (Fig. 2B,D,F). In addition, *Etv5* and *Dusp6* expression was notably upregulated in the surrounding neural crest (compare Fig. 2C,E with 2D,F).

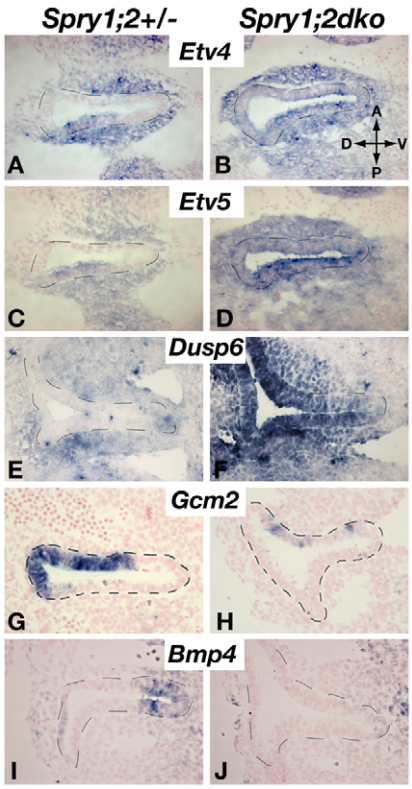

Fig. 2. Deregulated FGF signalling and delayed patterning events in the third pouch in the absence of *Spry1* and *Spry2*. (**A**,**B**) *Etv4* expression in sagittal sections through the third pouch of *Spry1;2*$^{+/-}$ control (A) and *Spry1;2dko* (B) mouse embryos at E10.5. (**C**,**D**) *Etv5* expression in sagittal sections through the third pouch of *Spry1;2*$^{+/-}$ control (C) and *Spry1;2dko* (D) embryos at E10.5. (**E**,**F**) *Dusp6* expression in sections of a similar plane to those in A and B confirming the expanded and increased FGF signalling in the third pouch at E10.5. (**G**,**H**) *Gcm2* expression revealing the presumptive parathyroid domain in the dorsal-anterior third pouch at E10.5. (**I**,**J**) *Bmp4* expression indicating the presumptive thymus domain in the ventral portion of the third pouch. The pharyngeal pouch is outlined by dashed lines. A, anterior; D, dorsal; P, posterior; V, ventral.

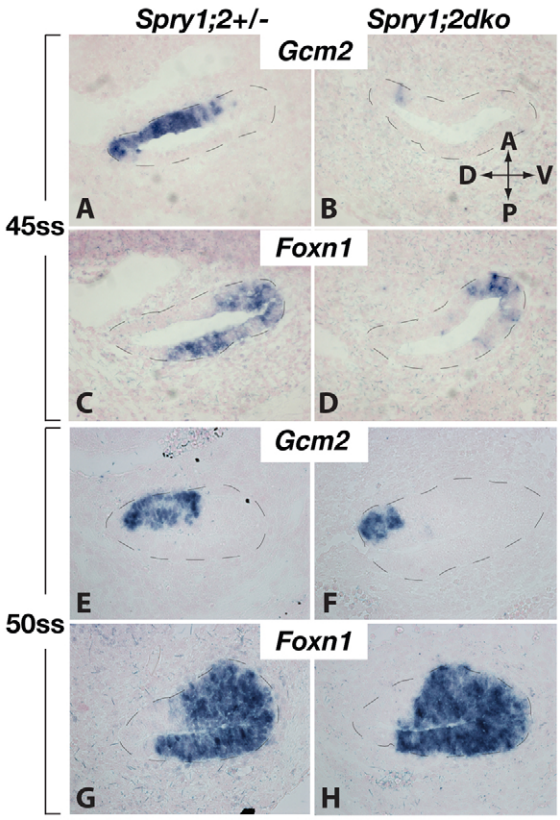

Fig. 3. Third pouch patterning defects in sprouty-deficient mouse embryos. (**A-D**) In situ hybridisation for *Gcm2* (A,B) and *Foxn1* (C,D) mRNA on adjacent sagittal sections through the third pharyngeal pouch of control (A,C) and mutant (B,D) embryos at E11.5 (45 ss). (**E-H**) *Gcm2* and *Foxn1* expression in adjacent sagittal sections through the third pouch of 50 ss control (E,G) and mutant (F,H) embryos. The pharyngeal pouch is outlined by dashed lines. A, anterior; D, dorsal; P, posterior; V, ventral.

These data are consistent with a model in which the characteristic pattern and level of FGF signalling in the third pouch endoderm is maintained by the localised inhibition by sprouty proteins.

Abnormal gene expression patterns in sprouty-deficient third pharyngeal pouches

Our data thus far suggested that *Spry1* and *Spry2* were essential for maintaining the pattern of FGF signalling in the third pouch endoderm at E10.5, i.e. at the time when the third pharyngeal pouch becomes subdivided into prospective *Bmp4*+ thymus and *Gcm2*+ parathyroid domains (Gordon and Manley, 2011). To determine whether the changes in FGF signalling in sprouty-deficient embryos were associated with altered patterns of thymus and parathyroid development, *Gcm2* and *Bmp4* gene expression was analysed at E10.5. In normal embryos, *Gcm2* expression is initiated in a region of endoderm encompassing the second and third pouch at E9.5 and becomes restricted to a defined region in the anterior-dorsal third pouch by E10.5 (Fig. 2G) (Gordon et al., 2001). Only a few isolated *Gcm2*+ cells could be detected in the third pouch in E10.5 *Spry1;2dko* embryos (Fig. 2J). *Bmp4* expression identifies the ventral pouch region that is fated to turn on *Foxn1* expression and become the thymus (Fig. 2I). *Bmp4* expression was downregulated in the ventral pouch in *Spry1;2dko* embryos (Fig. 2J). These observations suggest that the initiation of the patterning events that subdivide the third pouch into molecularly distinct regions is disrupted in *Spry1;2dko* embryos.

To understand the consequences of these early alterations in gene expression on the progression of thymus/parathyroid development, we followed *Gcm2* expression and the initiation of *Foxn1* expression further during development. At the 45 somite stage (ss), *Gcm2* expression was still seen in only a few isolated cells in the mutant embryos compared with the robust expression observed in controls (Fig. 3A,B). By this stage, *Foxn1* expression is visible in control embryos in the ventral pouch and has spread further along the posterior pouch towards the dorsal *Gcm2*+ domain (Fig. 3C), as described previously (Gordon et al., 2001). We find that *Foxn1* expression is induced in mutant embryos, albeit in fewer cells, which seem to be localised primarily in the ventral pouch with little evidence of spreading towards the dorsal side (Fig. 3D). By the 50

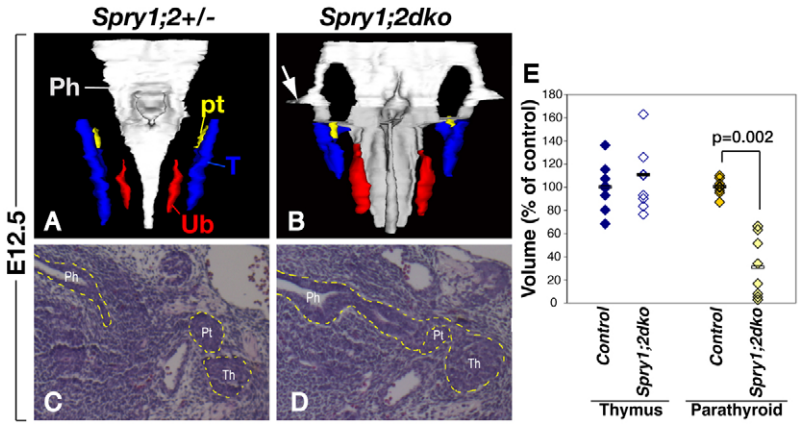

Fig. 4. Pharyngeal gland phenotypes in *Spry1;2dko* mouse embryos. (**A**,**B**) 3D reconstruction of structures in the pharyngeal region of E12.5 control (*Spry1*$^{+/-}$;*Spry2*$^{+/-}$, A) and *Spry1;2dko* mutant (*Spry1*$^{-/-}$;*Spry2*$^{-/-}$, B) embryos as seen from a ventral view. Note the attachment of the thymus (T, blue), parathyroid (pt, yellow) and ultimobranchial bodies (Ub, red) to the pharynx (Ph, grey) in the mutant, as well as the smaller size of the parathyroids and enlarged ultimobranchial bodies. The position where the second and third endodermal pouches remain attached to the second ectodermal cleft is indicated by an arrow in B. (**C**,**D**) H&E-stained sections from the embryos shown in A,B, with the pharynx, parathyroid and thymus outlined in yellow and annotated. Note the failure of the thymus and parathyroid to separate from the pharynx in D. (**E**) Volumes of thymi and parathyroids relative to the mean size of control glands at E12.5. Each symbol corresponds to an individual gland.

ss, a distinct *Gcm2*+ region in the correct anterior-dorsal position of the common thymus/parathyroid primordium was present in the mutant embryos (Fig. 3F). However, the size of the *Gcm2*+ domain was reduced compared with control embryos (Fig. 3E,F). By contrast, the *Foxn1*+ domain in the ventral-posterior pouch of the mutant embryos appeared no different in terms of size and intensity of expression compared with controls (Fig. 3G,H). These data suggest that *Foxn1* expression is delayed in *Spry1;2dko* embryos at early stages of development, but that this expression eventually recovers to form a normal thymus primordium by the 50 ss. We infer from these data that the reduced expression of *Gcm2* and the formation of a small *Gcm2*+ domain in the third pouch by E11.75 is likely to result in parathyroid hypoplasia, whereas alterations in *Foxn1* expression are unlikely to have functional consequences for thymus development.

Glandular and pharyngeal defects in *Spry1;2dko* embryos

A prediction from our gene expression analyses was that a normal thymus primordium would develop in *Spry1;2dko* embryos, whereas parathyroid primordia would be hypoplastic. To determine the consequences of sprouty gene deletion on thymus and parathyroid development, serial sections through E12.5 mutant and control embryos were reconstructed in three dimensions and the relative positions and sizes of the thymus and parathyroid glands were compared (Fig. 4A,B). In agreement with our prediction, a comparison of the relative sizes of the individual primordia (Fig. 4E) revealed that the parathyroid primordia were significantly hypoplastic in mutants compared with controls (31% of control, n=4, P=0.002), confirming that the smaller *Gcm2*+ domain at E11.5 (Fig. 3F) resulted in parathyroid hypoplasia. The thymus glands were of normal size (111% of control, n=4, P=0.2), indicating that the recovery of normal *Foxn1* expression by the 50 ss (Fig. 3H) was sufficient to allow for normal thymus development up to E12.5.

A number of additional defects were evident in *Spry1;2dko* embryos upon 3D reconstruction (Fig. 4A,B) of histological sections (Fig. 4C,D). First, the organ primordia derived from the third and fourth pouches remained attached to the pharynx. In addition, the third pouch-derived endoderm also remained attached to the ectoderm (Fig. 4B, arrow). The pharynx itself was wider than in controls, and all embryos examined had tracheo-oesophageal fistula. These data indicate that *Spry1* and *Spry2* are required for normal parathyroid organogenesis as well as for separation of organ primordia from the pharynx.

Sprouty loss of function inhibits cell death associated with the detachment of organ primordia from the pharynx

A previous study reported that the apoptotic death of endodermal cells at the junction between the thymus primordium and the pharynx at E11.75 (47-50 ss) was associated with the detachment of the thymus from the pharynx (Gordon et al., 2004). We therefore hypothesised that the failure of organ primordia to detach from the pharynx in the *Spry1;2dko* mutants might be due to defects in the initiation of programmed cell death. To test this hypothesis, apoptotic cells were visualised in control embryos by staining third pharyngeal pouch sections with antibodies to cleaved caspase-6. No apoptosis was detected in the third pouch of E10.5 embryos (34 ss, data not shown). However, a population of apoptotic cells was located in the dorsal half of the posterior pouch, close to the junction between the pouch and pharynx in the third pouch endoderm a few hours later at the 38-40 ss (E10.75) (Fig. 5A,C). This region corresponded to the domain where high levels of *Spry1* and *Spry2* genes were expressed (compare Fig. 5C with similar sections in Fig. 1G,H). These observations are in agreement with previous data showing that the primary site of attachment of both primordia to the pharynx is at the site between the *Gcm2*+ and *Foxn1*+ domains (Gordon and Manley, 2011).

To test whether the loss of sprouty genes prevented apoptosis in the third pouch, we also stained sections from *Spry1;2dko* pouches with an antibody to cleaved caspase-6. Cell death was reduced or absent in the dorsal-posterior pouches of mutant embryos at E10.75 (Fig. 5B,D), suggesting that apoptosis was indeed sensitive to the level of FGF signalling. We also observed apoptotic cells in the junction between the thymus primordium and the pharynx shortly

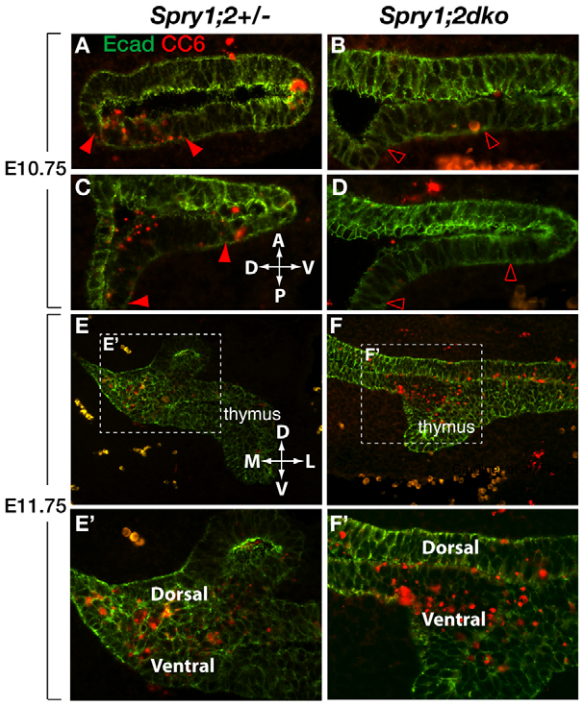

Fig. 5. Reduced apoptosis in the third pouch in the absence of *Spry1* and *Spry2*. (**A-D**) Sagittal sections through the third pouch at E10.75 at mid (A,B) and medial (C,D) pouch levels stained with antibodies to E-cadherin (Ecad, green) to visualise the endoderm and cleaved caspase 6 (CC6, red) to visualise apoptotic cells. Note the high number of apoptotic cells in the posterior-dorsal pouch endoderm in control embryos (area between red arrowheads in A and C) and the absence of apoptosis in the corresponding region of mutant embryos (area between open arrowheads in B and D). (**E**,**F**) Coronal sections through E11.75 embryos stained described for A-D. Note the apoptotic cells at the junction between the pharynx and thymus primordium in control (E) and mutant (F) embryos. (**E′**,**F′**) High magnification views of the boxed areas in E and F, respectively. The dorsal and ventral sides of the pouch endoderm are labelled. A, anterior; D, dorsal; L, lateral; M, medial; P, posterior; V, ventral.

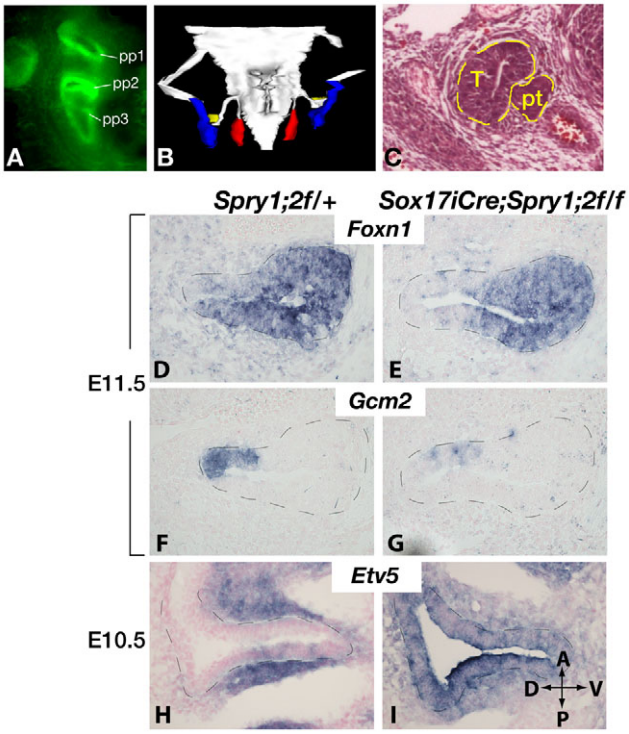

Fig. 6. *Spry1* and *Spry2* are required in the pharyngeal endoderm. (**A**) Green fluorescence in the pharyngeal pouches of an E10.5 *Sox17iCre;RosaYFP* embryo confirming Cre activity specifically in the pharyngeal pouch endoderm. A lateral view of the embryo is shown with the pharyngeal pouches 1-3 (pp1-pp3) indicated. (**B**,**C**) 3D reconstruction (B) and H&E-stained sections (C) from an E12.5 embryo in which *Spry1* and *Spry2* had been deleted specifically from the pharyngeal endoderm using *Sox17iCre*. pt, parathyroid; T, thymus. (**D-G**) *Foxn1* and *Gcm2* expression domains revealed by in situ hybridisation on sagittal sections through third pouches at E11.5. Note the smaller *Gcm2* domain in the mutant (compare G with F). (**H**,**I**) *Etv5* expression is expanded and upregulated in the mutant third pharyngeal pouch at E10.5. The pharyngeal pouch is outlined by dashed lines in D-I. A, anterior; D, dorsal; P, posterior; V, ventral.

before separation in control embryos at E11.75 (47-50 ss) (Fig. 5E,E′) as previously reported (Gordon et al., 2004). Interestingly, although apoptotic cells could be detected in this region in *Spry1;2dko* embryos, these cells were mainly observed in the ventral, but not the dorsal, side of the epithelial bridge between the pharynx and thymus in *Spry1;2dko* embryos (Fig. 5F,F′). These observations suggest that the loss of sprouty genes prevents normal programmed cell death in the dorsal-posterior pouch endoderm. Furthermore, although cell death is evident in the region between the organ primordia and the pharynx at E11.75 in sprouty-deficient embryos, the distribution of apoptotic cells in these mutants indicates that an abnormal apoptotic programme is most likely to be the cause of persistent attachment to the pharynx.

Spry1 and *Spry2* expression in the pharyngeal endoderm is essential for normal thymus and parathyroid development

Sprouty genes are not expressed in the pharyngeal endoderm alone and defects in early thymus and parathyroid organogenesis in *Spry1;2dko* mutants might be due to the combined affect of deleting these genes in several different cell types in the third pharyngeal arch (Fig. 1G,H). To determine whether the loss of sprouty genes in the pharyngeal endoderm alone could recapitulate some of the phenotypes in embryos that lack *Spry1* and *Spry2* in all tissues, mutants in which these genes were specifically removed from the pharyngeal endoderm using a *Sox17-iCre* line were produced (Engert, S. et al., 2009; Simrick et al., 2011). Cre activity in the pharyngeal pouch endoderm was confirmed by visualising the green fluorescent reporter in *Sox17-iCre;RosaYFP* mice (Srinivas et al., 2001) (Fig. 6A). *Sox17-iCre;* $Spry1^{flox/flox}$;$Spry2^{flox/flox}$ conditional mutants exhibited similar phenotypes to *Spry1;2dko* mutants at E12.5. Parathyroids were hypoplastic (43% of controls, n=3, P=0.004), thymus volumes were not statistically different from controls, and these organs failed to separate from the pharynx and were localised in abnormal orientations relative to each other (Fig. 6B,C). Analysis of *Gcm2* and *Foxn1* expression at E11.5 also demonstrated a smaller *Gcm2*+ domain (Fig. 6D-G). *Etv5* expression was again upregulated throughout the pouch endoderm at E10.5 (Fig. 6H,I). These data indicate that the function of sprouty genes as FGF inhibitors in the pouch endoderm is essential for establishing normal pouch patterning and organ separation from the pharynx.

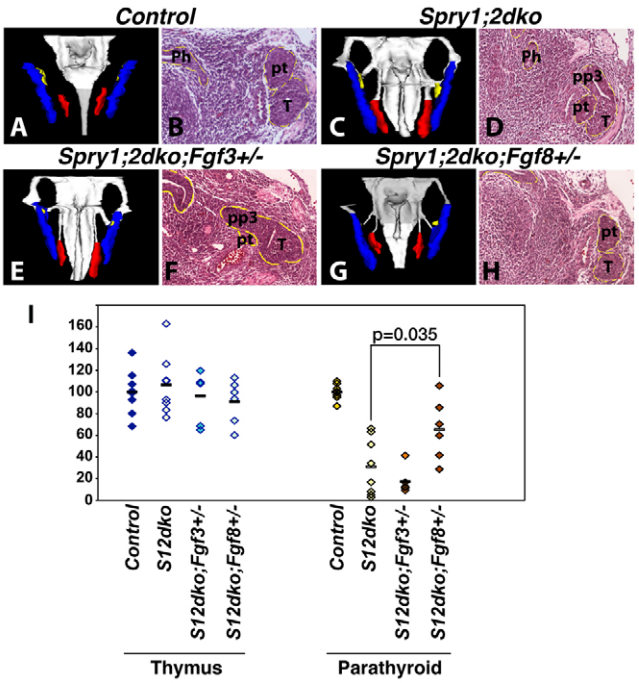

Fig. 7. *Fgf8* heterozygosity can partially rescue the sprouty-deficient phenotype. (**A**,**C**,**E**,**G**) 3D reconstructions of the pharyngeal region in E12.5 embryos of the indicated genotypes. (**B**,**D**,**F**,**H**) H&E sections through these embryos showing the thymus (T), parathyroid (pt), pharynx (Ph) and remaining epithelial attachment to the third pharyngeal pouch (pp3) in some embryos. Note the increased size of the parathyroid in H compared with D and F, and the correct position dorsal to the thymus in H compared with the abnormal position medially to the thymus in D and F. (**I**) Thymus and parathyroid volumes relative to control glands. Note the significant rescue of parathyroid hypoplasia in *Spry1;2dko* embryos heterozygous for *Fgf8*. Each symbol corresponds to an individual gland.

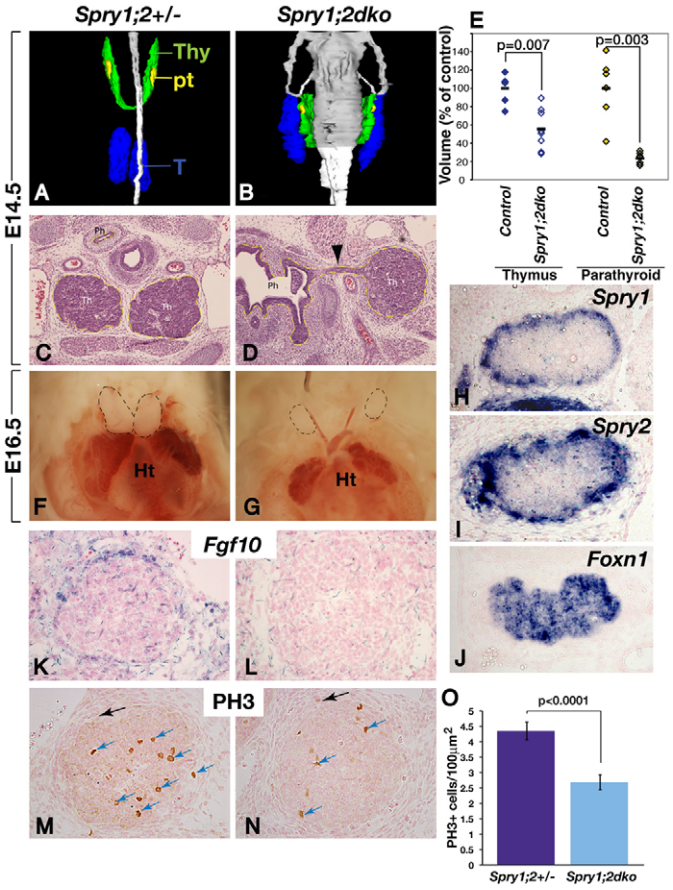

Fig. 8. Sprouty deletion results in reduced *Fgf10* expression in the thymus capsule and reduced proliferation of the thymus primordium after E12.5. (**A**,**B**) 3D reconstructions of the pharyngeal regions in E14.5 embryos, confirming the failure of the thymus (T, blue) and parathyroids (pt, yellow) to detach from the pharynx. The thyroid (Thy, green) is also represented. (**C**,**D**) H&E sections from the embryos shown in A and B. Note the epithelial bridge (arrowhead) linking the thymus (Th) with the pharynx (Ph) in D. (**E**) Volumes of thymi and parathyroids relative to the mean size of control glands at E14.5. Each symbol corresponds to an individual gland. (**F**,**G**) Photographs of the open chest cavities of E16.5 embryos with the thymi outlined by dashed lines. Note the abnormal position of the hypoplastic thymi in G. Ht, heart. (**H-J**) In situ hybridisation of *Spry1*, *Spry2* and *Foxn1* on adjacent sagittal sections through the E13.5 thymus primordium. Note the expression of *Spry1* and *Spry2* in the capsule surrounding the *Foxn1*+ thymus primordium. (**K**,**L**) *Fgf10* expression in the capsule surrounding a control thymus primordium (K) is lost in the mutant thymus primordium (L) at E13.5. (**M**,**N**) Phospho-histone H3 (PH3) immunohistochemistry to label proliferating cells (blue arrows) in E13.5 thymus primordia. Note the presence of polarised thymus capsule cells (black arrows) in both control and mutant sections despite the reduced *Fgf10* expression in the latter. (**O**) Quantification of PH3+ proliferating cells in thymus primordia of control and *Spry1;2dko* embryos. Error bars represent s.e.m.

Sprouty genes function as antagonists of Fgf8 signalling during thymus/parathyroid development

The results presented so far suggest that sprouty genes function to restrict FGF signalling in the third pouch endoderm during thymus/parathyroid development. However, as sprouty proteins act intracellularly to antagonise conserved signalling pathways that are utilised downstream of many receptor tyrosine kinases (Mason et al., 2006), the possibility that the phenotypes in *Spry1;2dko* embryos were due to hyperactive signalling downstream of receptor tyrosine kinases (RTKs) other than FGF receptors remained. We therefore tested whether genetic reduction of *Fgf3* or *Fgf8*, both expressed in the third pouch at E10.5 (Fig. 1) and reported to be involved in thymus organogenesis (Frank et al., 2002; Aggarwal et al., 2006), could rescue any of the observed phenotypes, which would indicate that hyperactive FGF signalling is responsible for that particular defect. *Fgf3* heterozygosity had no effect on any of the phenotypes in *Spry1;2dko* embryos (Fig. 7A-F,I). By contrast, halving the *Fgf8* gene dosage resulted in a partial rescue of the parathyroid and separation defects at E12.5. The parathyroids in *Spry1;2dko,Fgf8*$^{+/-}$ embryos were consistently found in the correct position dorsal to the thymus (n=6/6), had separated from the thymus primordia and were significantly larger than parathyroids in littermate *S1;2dko* mutants, although still hypoplastic compared with controls (65% of controls, compared with 25% in *Spry1;2dko* littermates, n=3, P=0.03) (Fig. 7G-I). In addition, three out of six primordia examined had separated from the pharynx. Taken together, these observations indicate that reduced Fgf8 levels moderated the effects of sprouty mutants, suggesting that sprouty proteins inhibited signalling downstream of

Fgf8 during third pouch patterning. We conclude that increased Fgf8 signalling is at least partially responsible for the separation defects and parathyroid hypoplasia in *Spry1;2dko* embryos.

Thymus hypoplasia in sprouty-deficient embryos is associated with reduced *Fgf10* expression in the thymus capsule

Analysis of E14.5 embryos (Fig. 8A,B,E) indicated that the parathyroid hypoplasia observed in sprouty-deficient embryos at E12.5 failed to recover during development (mutant parathyroids were 23% the size of controls, n=4, P=0.003). Surprisingly, sprouty mutant thymi were also hypoplastic by E14.5 (56% of control, n=4, P=0.007), suggesting a role for sprouty genes in thymus growth between E12.5 and E14.5 (Fig. 8E). As a consequence of the physical attachment of the thymus to the pharynx (Fig. 8D), the hypoplastic thymus lobes were present in ectopic locations in the neck adjacent to the pharynx, compared with normal thymi, which by this stage had migrated to positions above the heart (Fig. 8A,B). Hypoplastic thymi located in ectopic positions in the neck were clearly visible in embryos just before birth (Fig. 8F,G).

As previous studies have suggested that FGFR2(IIIb) ligands such as Fgf10 are responsible for driving the rapid expansion of the thymus primordium after E12.5 (Ohuchi et al., 2000; Revest et al., 2001), we investigated whether sprouty genes were still expressed in the thymus primordium at this time of growth. In situ hybridisation experiments demonstrated that *Spry1* and *Spry2* genes were expressed specifically in cells of the thymic capsule that surrounds the thymus primordium at E13.5 (Fig. 8H,I) and not in the thymus itself (marked by *Foxn1* expression in Fig. 8J). Comparison of *Fgf10* expression in control and *Spry1;2dko* embryos at E13.5 revealed a clear reduction in *Fgf10* expression in the thymus capsule of sprouty-deficient embryos at E13.5 (Fig. 8K,L). To determine whether the loss of *Fgf10* expression was associated with reduced proliferation of thymic epithelial cells, cells in mitosis were quantified after staining with an antibody to phospho-histone H3. *Spry1;2dko* thymic primordia contained significantly fewer mitotic cells compared with control thymi at E13.5 (Fig. 8M-O). These observations suggest that thymus hypoplasia in *Spry1;2dko* embryos is due to reduced thymus growth as a result of the loss of expression of the thymic epithelial mitogen Fgf10 in the thymic capsule at E13.5 of development.

DISCUSSION

Our analysis of thymus and parathyroid development in sprouty-deficient embryos has revealed several key developmental processes that require tight regulation of FGF signalling. Inhibition of FGF signalling is required for the normal initiation of *Gcm2* expression and the establishment of a normal-sized parathyroid primordium. Although deregulated FGF signalling is also associated with defects in the initiation of *Bmp4* and *Foxn1* expression, these abnormalities appear to recover such that a normal thymus primordium initially forms. However, we produce evidence for a continued requirement for sprouty gene function during later stages of thymus expansion, such that the loss of sprouty genes is associated with loss of *Fgf10* expression in the thymus capsule, reduced TEC proliferation and thymus hypoplasia. Sprouty gene function is also required for the regulation of FGF signalling during organ detachment from the pharynx.

Parathyroid development

Our data indicate that increased FGF signalling in the absence of *Spry1* and *Spry2* results in diminished induction of *Gcm2* expression resulting in the formation of a small *Gcm2*+ domain by E11.5 (Fig. 9). These observations suggest that defects in *Gcm2* expression underlies the parathyroid hypoplasia observed in *Spry1;2dko* mutants. Currently, the exact mechanism by which *Gcm2* expression is repressed in sprouty mutants is not known. Hyperactive FGF signalling might repress *Gcm2* transcription either directly, or by inhibiting the expression or activity of upstream transcriptional regulators of *Gcm2*, such as Gata3 (Grigorieva et al., 2010). Previous studies have shown that Shh signalling induces *Gcm2* expression (Moore-Scott and Manley, 2005), raising the possibility that the effect of sprouty gene deletion on *Gcm2* expression might be indirect through, for example, the inhibition of Shh signalling, as recently reported in the developing cerebellum (Yu et al., 2011).

Thymus organogenesis

We found that *Bmp4* expression, which marks the presumptive thymus domain, is reduced in *Spry1;2dko* embryos (Fig. 9). Changes in FGF signalling are documented to affect *Bmp4* expression in other developmental contexts, such as forebrain patterning, raising the possibility that similar mechanisms might be responsible for these changes during third pouch patterning (Storm et al., 2006). *Bmp4* has been implicated in the regulation of *Foxn1* expression in mouse and chick and reduced BMP signalling results in thymus hypoplasia in the mouse (Ohnemus et al., 2002; Neves et al., 2012; Bleul and Boehm, 2005; Soza-Ried et al., 2008; Gordon et al., 2010). These observations suggested the possibility that the reduced *Bmp4* expression in the sprouty-deficient pouch at E10.5 might result in thymus hypoplasia in *Spry1;2dko* embryos. However, despite these early alterations in *Bmp4* expression, and a corresponding delay in the initiation of *Foxn1* expression in sprouty-deficient embryos, *Foxn1* expression appears largely normal by the 50 somite stage and thymus primordia are of normal size by E12.5 of development. These observations suggest that an early defect in *Foxn1* expression can recover to allow for normal development to ensue. A similar example of a recovery of an early defect in tooth development due to deficient BMP signalling was recently reported, suggesting that this phenomenon of 'developmental stalling' might be a common feature of BMP-regulated developmental processes (Miletich et al., 2011).

Curiously, thymi were significantly smaller by E14.5, suggesting that the loss of sprouty genes affected thymus expansion between E12.5 and E14.5. As Fgf10/Fgfr2b signalling has been shown to be required for the growth of the thymus primordium after E12.5, one might have expected the opposite phenotype, i.e. thymus overgrowth when deleting FGF antagonists. However, we find that sprouty genes are not expressed in TECs and that their deletion did not result in FGF hyper-responsive, over-proliferating TECs owing to the loss of FGF inhibition in TECs. Instead, sprouty genes are expressed in the surrounding thymus capsule at E13.5 and the loss of sprouty genes was associated with downregulation of *Fgf10* expression and reduced TEC proliferation. The downregulated expression of *Fgf10* might be due to a direct repression of *Fgf10* gene expression by FGF signalling. A previous study has shown that the Ets transcription factor Pea3, the product of the *Etv4* gene, which is induced by FGF signalling, can bind directly to an *Fgf10* promoter and repress *Fgf10* transcription (Chioni and Grose, 2009). The identification of capsule-specific *Fgf10* gene regulatory elements and their regulation by FGF signalling should provide further insights into this phenomenon. We also considered the possibility that the loss of *Fgf10* expression might be due to general defects in the thymus capsule. *Fgf10* expression in the neural crest surrounding the third pouch was unaffected at E10.5 (data not

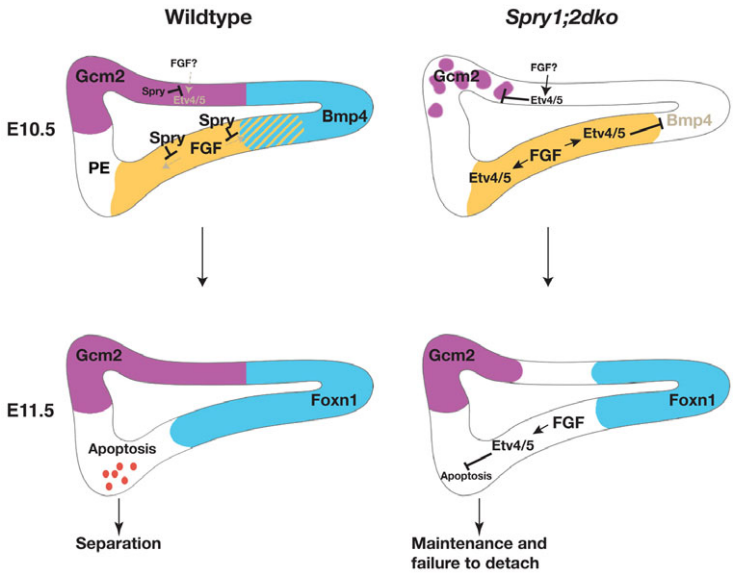

Fig. 9. Model diagram depicting changes in gene expression in the third pharyngeal pouch upon sprouty gene deletion. At E10.5, *Gcm2* and *Bmp4* are expressed in complementary domains in wild-type embryos. FGF ligands expressed in the posterior pouch endoderm do not induce high expression of downstream genes such as *Etv4/5* in the endoderm owing to the inhibition of FGF signalling by high levels of sprouty (Spry). PE indicates the dorsal pharyngeal endoderm that lines the pharynx where the pouch-derived organs will detach from the pharynx. In Spry-deficient embryos, high levels of *Etv4/5* are expressed in the endoderm, *Bmp4* expression is downregulated and few *Gcm2*+ cells are present. By E11.5, complementary *Gcm2*+ (parathyroid) and *Foxn1* (thymus) domains are present in the third pouch of wild-type embryos. Apoptotic cells (red) are evident in the posterior-dorsal pouch endoderm and pre-figures the separation of the organ primordia from the pharynx. In *Spry1;2dko* embryos, the *Gcm2* domain is smaller resulting in parathyroid hypoplasia. A delay in Foxn1 expression and spreading along the posterior pouch at the 45 ss eventually recovers to form a normal *Foxn1*+ domain. Apoptotic cells are absent from the posterior-ventral pouch in *Spry1;2dko* embryos suggesting that increased FGF signalling in this region prevents apoptosis and the detachment of organ primordia.

shown), ruling out severe defects in early neural crest development. Furthermore, the thymus capsule appears histologically normal in sprouty mutants at E13.5 (black arrows in Fig. 8M,N). Nevertheless, we cannot at this stage rule out the possibility that subtle changes in the differentiation state of these cells could account for a reduced ability to express *Fgf10*.

Organ detachment from the pharynx

A striking consequence of deleting sprouty genes during development is the failure of organ primordia to detach from the pharynx and from each other. This phenotype was preceded by the loss of apoptosis normally present in the posterior-dorsal pouch from E10.75. This apoptotic domain is localised to a region between the prospective *Gcm2*+ parathyroid and *Bmp4*+ thymus domains in the dorsal half of the posterior pouch endoderm at E10.5 where the pouch is attached to the non-pouch pharyngeal endoderm (PE in Fig. 9). We have detected the expression of *Fgf3*, *Fgf8* and *Fgf15* in and adjacent to this region, suggesting that the regulation of FGF signalling might be intimately linked to the separation process. The observation that low levels of FGF signalling in the posterior-dorsal pouch are associated with apoptosis during normal development is in agreement with previous demonstrations that FGF signalling is required to maintain cell survival in several developmental contexts including the brain (Basson et al., 2008; Paek et al., 2009), limb (Sun et al., 2002), kidney (Grieshammer et al., 2005) and olfactory system (Kawauchi et al., 2005). In all these examples, abnormally reduced FGF signalling is associated with aberrant cell death and loss of tissues or structures. In the present study, we show that the localised inhibition of FGF signalling by sprouty genes is essential for correct morphogenesis by allowing the formation of a discrete domain of cell death in the pouch endoderm. Furthermore, our data suggest that the surviving *Foxn1;Gcm2*-negative cells persist as an epithelial bridge between the pharynx and the thymus/parathyroid primordia in *Spry1;2dko* embryos, easily observed a day later, at E11.75. We propose that these changes in morphology and apoptosis, arising due to increased FGF signalling, are collectively responsible for the failure of organ detachment.

A recent study showed that the deletion of an essential intracellular mediator of FGF signalling, Frs2α, is associated with a general failure of organ detachment from the pharynx (Kameda et al., 2009). Superficially, this observation appears to be at odds with our findings. However, previous studies focusing on nervous system development have found that increasing or decreasing FGF signalling could have similar effects on cell survival (Storm et al., 2003). It will be interesting to determine whether sprouty gene expression is sufficiently downregulated in the Frs2α mutants to prevent apoptosis in the posterior pouch endoderm in an analogous fashion to the findings in the forebrain.

FGF signalling and thymus/parathyroid organogenesis

The downstream, transcriptional effectors of FGF signalling that are responsible for mediating the various phenomena described in this manuscript are not known. Likely candidates are the Ets family transcription factors encoded by the *Etv4* and *Etv5* genes (Fig. 9), as recent studies have shown that these factors are the physiological effectors of FGF signalling in other developmental systems (Mao et al., 2009). As far as we are aware, thymus/parathyroid development has not been investigated in mutants that lack *Etv4* and/or *Etv5*. Although it is difficult to predict the thymus/parathyroid phenotypes of these mutants at this stage, we predict that the inducible overexpression of *Etv4* or *Etv5* would result in similar effects on thymus and parathyroid development as we describe here for sprouty-deficient embryos.

In conclusion, we have identified several roles for FGF antagonists of the sprouty gene family during thymus and parathyroid organogenesis. The inhibition of FGF signalling by sprouty proteins is crucial for normal *Gcm2* induction and parathyroid size, apoptosis required for organ separation from the thymus, and expansion of the thymus primordium by proliferation. This study suggests that a full understanding of these developmental processes will require studies aimed at elucidating the role of FGF signalling and the integration of FGF signalling with other developmental pathways.

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Competing interests statement
The authors declare no competing financial interests.

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