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Developmental Biology 278 (2005) 323–335

DEVELOPMENTAL BIOLOGY

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Differential expression of Sonic hedgehog along the anterior–posterior axis regulates patterning of pharyngeal pouch endoderm and pharyngeal endoderm-derived organs

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Received for publication 27 January 2004, revised 20 September 2004, accepted 25 October 2004
Available online 2 December 2004

Abstract

Previous studies have implicated Sonic hedgehog (Shh) as an important regulator of pharyngeal region development. Here we show that *Shh* is differentially expressed within the pharyngeal endoderm along the anterior–posterior axis. In $Shh^{-/-}$ mutants, the pharyngeal pouches and arches formed by E9.5 and marker expression showed that initial patterning was normal. However, by E10.5–E11.0, the first arch had atrophied and the first pouch was missing. Although small, the second, third, and fourth arches and pouches were present. The expression patterns of *Fgf8*, *Pax1*, and *Bmp4* suggested that pouch identity was abnormal at E10.5 and that Shh is a negative regulator of these genes in the pouches. Despite the loss of pouch identity and an increase in mesenchymal cell death, arch identity markers were expressed normally. Our data show that a Shh-dependent patterning mechanism is required to maintain pouch patterning, independent or downstream of arch identity. Changes in the distribution of *Bmp4* and *Gcm2* in the third pouch endoderm and subsequent organ phenotypes in $Shh^{-/-}$ mutants suggested that exclusion of *Shh* from the third pouch is required for dorsal–ventral patterning and for parathyroid specification and organogenesis. Furthermore, this function for Shh may be opposed by Bmp4. Our data suggest that, as in the posterior gut endoderm, exclusion of *Shh* expression from developing primordia is required for the proper development of pharyngeal-derived organs.

Keywords: Pharyngeal pouches; Pharyngeal arches; Endoderm; Parathyroid; Thymus; Sonic hedgehog; Bone morphogenetic protein 4; Homeobox transcription factors; Gcm2; Foxn1

Introduction

The pharyngeal region in vertebrates is a specialized arrangement of structures including the pharyngeal arches, pouches, and clefts, which become morphologically distinct as early as E8.0 in the mouse embryo (Graham, 2003; Graham and Smith, 2001). In mice, there are five arches arranged along the anterior–posterior axis, each of which is patterned to contribute to both morphological and functional structures of the face and neck. The arches persist until approximately E11.5–E12.0, when they broaden and flatten externally as they begin to form the neck of the embryo. Within each arch, several multipotent cell populations including the neural crest, mesoderm, endoderm, and ectoderm combine together in both a physical and regulatory manner to create the characteristic morphology of this region. All cell populations of the pharyngeal arches contribute to, or are the origin of, multiple specialized organs, vascular and neuronal tissues, as well as the muscular and skeletal components of the head and neck.

The endoderm-derived epithelial layer that lines the pharynx and the pharyngeal pouches is the origin of several organ primordia that are derived from the pharyngeal region. The thyroid primordium forms from the endoderm on the ventral midline of the second arch. The pouches of

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doi:10.1016/j.ydbio.2004.10.027

the posterior third and fourth arches form the primordia of the thymus and parathyroid, and the ultimobranchial bodies, respectively. The pharyngeal endoderm has been proposed to act as a signaling center for patterning of this region (Graham and Smith, 2001; Piotrowski and Nusslein-Volhard, 2000). Therefore, the precise patterning of this region is essential for the specification of these cells, and factors that are involved in this process can have a considerable impact on the proper morphology and function of multiple tissues.

Sonic hedgehog (Shh) is a secreted, lipid-modified glycoprotein that acts as a morphogen capable of migrating anywhere from 80 to 300 μm dependent upon the tissue (Gritli-Linde et al., 2001; Ingham and McMahon, 2001). *Shh* is expressed in several signaling centers, including the ZPA of the limb bud and the floor plate of the neural tube, and is essential for regionalization of these tissues. The Shh signaling pathway is activated through Smoothened (Smo) upon binding of Shh to the hedgehog receptor Patched (Ptc). The Shh signal is primarily mediated through zinc finger transcription factors Gli1, Gli2, and Gli3. Shh activity patterns tissues by generating domains of specialized cell types often in a concentration-dependent manner. Although the mechanism is not entirely clear, the hedgehog pathway manipulates the expression or activity of the Gli proteins such that differential and overlapping patterns emerge. This process then results in the induction or repression of target genes, thereby creating regions of multiple specified cell types within a tissue.

During the formation of specialized domains within a given tissue, polarity is often established by members of the bone morphogenetic protein (Bmp) and wingless (Wnt) families, which oppose either the activity or expression of *Shh* (Lee et al., 2001; Marcelle et al., 1997; Zhang et al., 2002). These signaling pathways can work cooperatively with and in opposition to each other and to the *Shh* pathway to sculpt tissues structurally as well as functionally. *Shh*, *Fgf8*, and *Bmp4* have proven to be important in pharyngeal region patterning as their loss or improper regulation of their activity results in hypoplastic or missing pharyngeal arch structures (Abu-Issa et al., 2002; Ahlgren and Bronner-Fraser, 1999; Bachiller et al., 2003; David et al., 2002; Frank et al., 2002; Ohnemus et al., 2002; Revest et al., 2001; Stottmann et al., 2001; Trokovic et al., 2003). The *Shh* mutant was reported to have small yet essentially normal pharyngeal arches by E9.5 (Chiang et al., 1996), and NCC-specific deletion of *Smo* showed that anterior arch development and jaw formation is dependent on a Shh survival signal (Jeong et al., 2004). The loss of *Fgf8* and the failure to properly regulate Bmp4 both result in the loss of the third and fourth arches and pouches and their derivatives (Abu-Issa et al., 2002; Jerome and Papaioannou, 2001). Just how these regulatory pathways interact to regulate the patterning of the pharyngeal region is as yet unknown.

Although the *Shh* mutant has an undeniably severe phenotype, it can provide valuable information concerning developmental mechanisms in mid to late gestation stage embryos (Chiang et al., 1999; Mahlapuu et al., 2001; Pepicelli et al., 1998; Ramalho-Santos et al., 2000; Shah et al., 2004; St-Jacques et al., 1998). In this report, we have investigated the role of Shh in mouse pharyngeal region development and pharyngeal organogenesis by analysis of the $Shh^{-/-}$ mutant mouse phenotype. We found that *Shh* and *Ptc1* gene expression along with the *Shh* mutant phenotype indicate that there is a higher dependence of anterior arch morphology on *Shh* activity. Our results suggest that multiple cell populations of the pharyngeal arches require Shh activity for survival and agree with previous findings that *Shh* is not required for initial formation and patterning of the pharyngeal region. We provide evidence that independent regulatory mechanisms establish or maintain pouch and arch identity, and that Shh acts predominantly as a repressor of key pharyngeal pouch identity genes including FgfB and Bmp4, during this process. Furthermore, our data suggest that opposing Shh and Bmp signals are required for subsequent patterning and organogenesis in the third pouch, as parathyroid development is absent in *Shh* mutant embryos, while the thymus domain and Bmp4 expression are expanded. Our results indicate multiple essential functions for Shh signaling in pharyngeal region patterning and organogenesis.

Materials and methods

Mice

Sonic hedgehog knockout mice were provided by Chin Chiang (Vanderbilt). Genotyping was performed as described (Chiang et al., 1996). The mice used were maintained on a 129(SvJ) by C57Bl/6 F1 genetic background. Control mice were staged according to the date of vaginal plug, somite number (E9.5, 20–25 somites; E10.25–E10.5, 30–35 somites; E10.5–E11.0, 35–40 somites), or morphology (including the limbs and pharyngeal arches for E11.5), according to published descriptions (Kaufman, 1992). As the Shh homozygous mutants have severe dismorphologies, they were staged primarily according to their wild type and heterozygous littermates. Swiss Webster (Taconic) embryos were used for the analysis of wild type expression patterns where indicated. We found no difference in *Shh* expression in Swiss Webster and C57Bl/6 mice.

Histology, 3D reconstruction, and scanning electron microscopy

For histology, $Shh^{-/-}$ embryos and wild-type littermates were collected at E9.5 (20–25 somites) and E10.5 (35–40 somites). Embryos were fixed in 4% paraformaldehyde (PFA) then dehydrated in a graded ethanol series, embedded in paraffin, sectioned, and stained with hematoxylin and eosin using standard methods. Digital images of serial

sections were reconstructed into a three-dimensional image using Surfdriver™ 3.5.3 software (Surfdriver).

For SEM, E9.5, E10.5, and E11.5 embryos were collected and staged as described above and fixed in 4% EM grade glutaraldehyde overnight at 4°C. These were dehydrated in a graded ethanol series and processed for SEM as described previously (Moore-Scott et al., 2003).

In situ hybridization

Whole mount and paraffin section in situ hybridizations were performed as described (Carpenter et al., 1993; Manley and Capecchi, 1995), using either Swiss Webster wild type or *Shh*$^{-/-}$ and littermate control embryos where indicated. Each probe was analyzed on a minimum of 2–3 embryos per stage. The *Hoxa3*, *Pax1*, *Hoxa2* (Manley and Capecchi, 1995); *Shh* (Echelard et al., 1993); *Fgf8* (Crossley and Martin, 1995), *Ptc1* (Goodrich et al., 1996); *Gli1*, *Gli2*, *Gli3* (Hui et al., 1994; Platt et al., 1997; Sasaki et al., 1999); *Gcm2*, *Foxn1* (Gordon et al., 2001); *Dlx3* (Clouthier et al., 2000) probes have been previously described. The *Hoxb1* probe was generated from a 300-bp *Pst*I–*Bam*HI fragment 3′ of the homeobox. Sections were counterstained with nuclear fast red.

Cell death and proliferation assays

Shh$^{-/-}$ mutant and control littermates were assayed for cell death by incubating E9.5 and E10.5 embryos with lysotracker red (Molecular Probes) for 30 min in serum-free culture medium as described (Moore-Scott et al., 2003). The embryos were fixed with 4%PFA, cleared with 1:2 benzyl alcohol:benzyl benzoate (BABB), and then visualized with confocal microscopy as described (Zucker et al., 2000). Cell proliferation was measured by indirect immunofluorescence in E9.5 and E10.5 Shh mutants and control littermates with anti-phosphohistone H3 antibody (Upstate Biotechnology) as described (Abu-Issa et al., 2002). Nuclei were visualized with DAPI and positive cells were counted using ImageJ software (developed at the U.S. National Institutes of Health; http://rsb.nih.gov/nih-image/) and quantitated as described (Jeong et al., 2004).

Results

Hedgehog activity is predominantly in the anterior pharyngeal region

As an initial step in studying the role of Shh in pharyngeal region development of mid-gestation stage embryos, we determined the expression patterns of *Shh* and its receptor *Ptc1* using paraffin section in situ hybridization analysis. *Ptc1* is upregulated in response to Shh signaling and is used an indicator of Shh activity (Hynes et al., 1997; Platt et al., 1997; Ruiz i Altaba et al., 2003; Sasaki et al., 1997). At E9.5, *Shh* and *Ptc1* were expressed in a restricted manner throughout the pharyngeal endoderm and surrounding arches (Fig. 1). The characteristic expression pattern of *Shh* was evident in the floorplate of the neural tube and notochord (Figs. 1A–D). Lower levels by comparison were visible in both the dorsal and ventral pharyngeal endoderm. The only exception to ventral endoderm expression is in the thyroid diverticulum at the level of the second pouch, where *Shh* expression was excluded (Fig. 1B). *Shh* expression was also not detected in the endoderm of the first (Fig. 1A), second (Fig. 1B), or third (Fig. 1C) pouches. *Shh* was expressed throughout the pharyngeal endoderm at the location where the fourth pouch will form, just below the third pouch and above the future laryngeo/tracheal groove (Fig. 1D). *Ptc1* expression was also restricted in the pharyngeal region at this stage (Figs. 1E–H). At E9.5, *Ptc1* was expressed within the endoderm and in the mesenchyme in close proximity to the *Shh*-expressing endoderm, but was downregulated in the most distal tips of the first and second pharyngeal pouches (Figs. 1E and F), and was not detected in the distal third pouch (Fig. 1G). At this stage, *Ptc1* expression is more extensive in the lateral arch mesenchyme of the first and second arches (Figs. 1E and F) than that of the more posterior third arch and the mesenchyme surrounding the future laryngeo/tracheal diverticulum (Figs. 1G and H).

At E10.5, *Shh* expression in the endoderm was more intense and expanded further into the first and second pouches (Figs. 1I and J) but remained restricted to the opening of the third and fourth pouches (Figs. 1K and L). *Ptc1* expression was expanded at this stage in the endoderm and the ventral mesenchyme (Figs. 1M–P). Although *Ptc1* was upregulated in the most lateral mesenchyme and in the ectodermal cleft of the first arch (Fig. 1M), it remained at a lower level in the lateral mesenchyme at the level of the second, third, and fourth pouches (Figs. 1N–P). By E11.5, the expression of both *Shh* and *Ptc1* had expanded further into the first and second pouches, although *Shh* expression was still excluded from the most distal tips (Figs. 1Q and R). However, even at this later stage, *Shh* was not detected in the third or fourth pouches (Figs. 1S and T). At this stage, *Ptc1* was expressed throughout the first and second pouches, surrounding mesenchyme and surface ectoderm of the first and second arches. However, *Ptc1* expression was still restricted from the most distal endoderm and mesenchyme of the third and fourth arches and pouches (Figs. 1U–X). As these are the locations of the epithelial primordia that give rise to the thymus and parathyroid and the ultimobranchial bodies, respectively, both *Shh* and *Ptc1* expression were restricted from regions that give rise to pharyngeal pouch-derived organ rudiments. These data show that *Shh* and *Ptc1* are differentially expressed, both temporally and spatially, in the pharyngeal region along the anterior–posterior (A–P) axis and suggest a higher level of Shh signaling in the anterior pharyngeal arches. Indian hedgehog (*Ihh*), although co-expressed with *Shh* in the

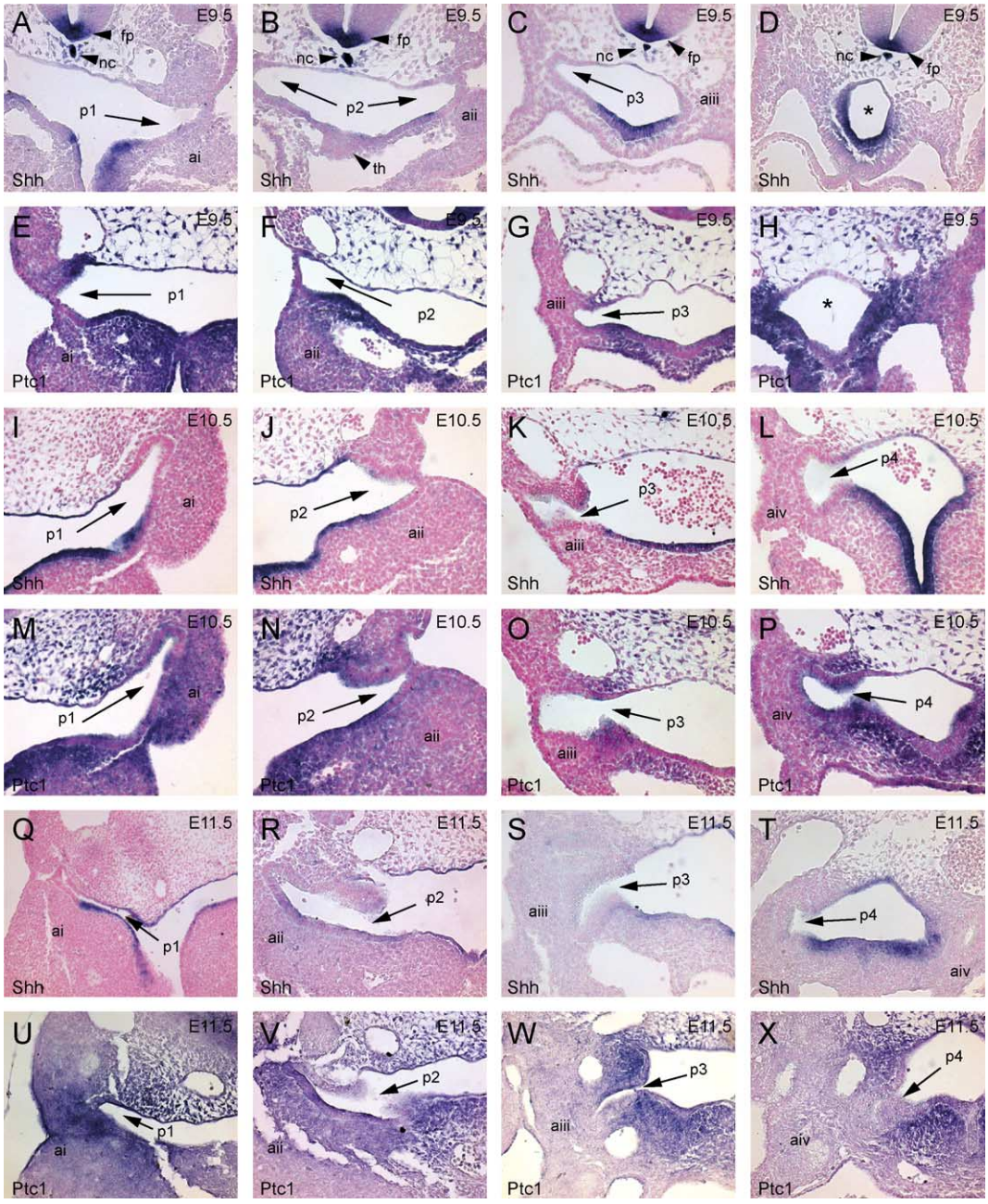

Fig. 1. Differential expression of *Shh* and *Ptc1* along the anterior to posterior axis in the pharyngeal region. Section in situ hybridization of *Shh* (A–D, I–L, Q–T) and *Ptc1* (E–H, M–P, U–X) in E9.5 (A–H), E10.5 (I–P), and E11.5 (Q–X) Swiss Webster embryos in the transverse plane. p1–4, pharyngeal pouches; aI–IV, pharyngeal arches; th, thyroid diverticulum; fp, floor plate; nc, notochord.

more posterior region of the gut, is not present in the pharyngeal gut endoderm and so does not play a role in pharyngeal endoderm patterning (data not shown; Bitgood and McMahon, 1995; Jeong, 2004).

Anterior/posterior arch patterning is unaffected in Shh$^{-/-}$ mutants

During early embryonic development, the Hox genes establish proper A/P patterning along the body axes (Hunt and Krumlauf, 1992). Shh has been shown to regulate the expression of Hox genes in the posterior hindgut and during early mesodermal patterning (Roberts et al., 1995). Therefore, we determined whether the absence of Shh would result in a loss of proper A–P patterning in the pharyngeal arches. We examined *Dlx3*, *Hoxa2*, and *Hoxa3*, which are homeobox genes expressed in multiple cell types and with distinctive patterns in the pharyngeal region that are indicative of arch identity (Fig. 2). *Dlx3* is expressed in the surface ectoderm and the underlying mesenchyme of the

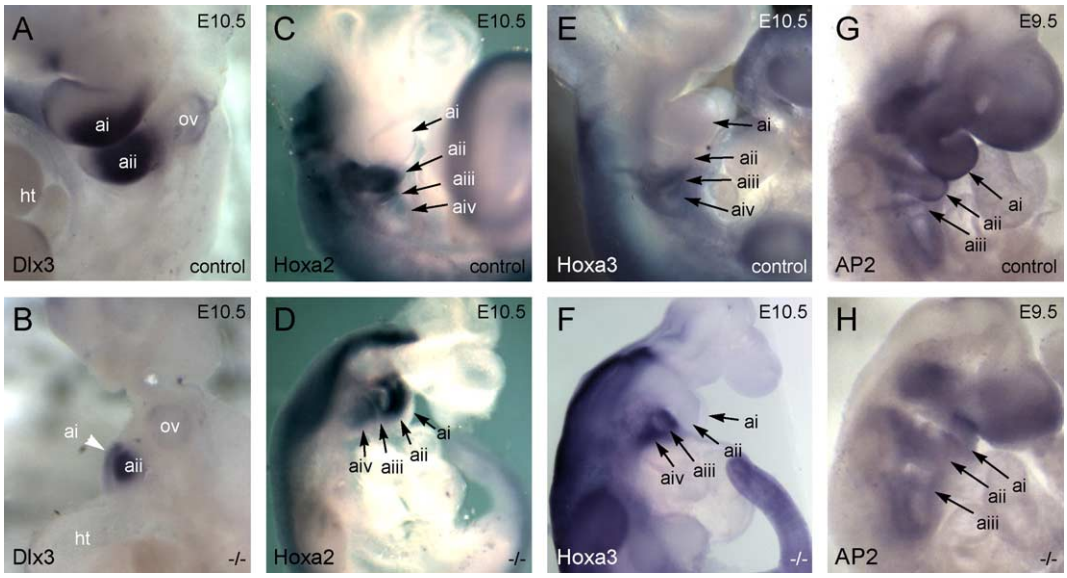

Fig. 2. Arch identity and neural crest migration are unaffected in E10.5 *Shh*$^{-/-}$ mutants. Whole mount in situ hybridization of the A/P patterning genes *Dlx3* (A and B), *Hoxa2* (C and D), *Hoxa3* (E and F) and the neural crest cell migration marker *AP2* (I and J). In E10.5 *Shh*$^{-/-}$ mutant embryo, *Dlx3* expression is visible in the second arch and in a section of cells located anterior to the second arch (A and B), identifying it as the atrophied first arch (see Figs. 3B and D in red). Both *Hoxa2* (D) and *Hoxa3* (F) are expressed normally in mutants (D and F) in comparison to control littermates (C and E). *AP2* is expressed properly showing that neural crest cells migrated normally into the correctly specified pharyngeal arches of both the control and *Shh*$^{-/-}$ mutant embryos at E9.5 (G and H). Note: head has been pulled back in B to better depict *Dlx3* staining in the first arch remnant indicated by the arrowhead. aI–III, arches.

posterior portion of the first and the second arch (Clouthier et al., 2000). Both *Hoxa2* and *Hoxa3* are expressed in the ectoderm, endoderm, mesoderm, and neural crest cells with anterior limits of expression in the second and third arches, respectively (Gaunt, 1987; Hunt et al., 1991; Manley and Capecchi, 1995). At E10.5, all of these markers were appropriately expressed in *Shh*$^{-/-}$ embryos. In control embryos, *Dlx3* was expressed in the first and second arches (Fig. 2A). In *Shh*$^{-/-}$ mutant embryos, *Dlx3* was still highly expressed in the second arch (Fig. 2B) and was detectable in the remnant of the first arch, indicating that despite its severely hypoplastic state, first arch identity was maintained (Fig. 2B, arrowhead). *Hoxa2* expression was normal in both control and mutant embryos, with its anterior limit and characteristically elevated expression in the second arch (Figs. 2C and D). *Hoxa3* was also expressed properly in both control and mutant embryos with its anterior boundary at the third arch (Figs. 2E and F). Expression of *AP2*, a marker for neural crest cells into the arches, was normal in E9.5 *Shh*$^{-/-}$ mutants, indicating that initial migration of neural crest cells was unaffected (Figs. 2I and J).

These results suggested that the first arch was dramatically reduced in size at E10.5, but was still present. This result was confirmed by SEM analysis of E10.5 and E11.5 embryos (Figs. 3A–D). Although the first arch was small, it was present, even at E11.5 (Figs. 3C and D). Taken together, these results suggest that *Shh* activity does not regulate A–P patterning genes in the pharyngeal region, and therefore does not appear to regulate arch identity. Shh is required, however, to maintain first arch morphology.

Altered expression of pharyngeal pouch markers in Shh mutants

To investigate the effect of Shh on endodermal patterning, we examined the expression of genes that are markers for pouch identity. *Fgf8* is essential for pharyngeal region development and is normally expressed in the endoderm of the second, third, and fourth pouches as well as the ectoderm of the clefts (Figs. 4A and C) (Abu-Issa et al., 2002). *Pax1* is expressed in the pharyngeal endoderm of the first, second, and third pouches and more weakly in the fourth (Figs. 4E and G) (Muller et al., 1996). At E9.5 (20–25 somites), both Fgf8 (Fig. 4B) and Pax1 (Fig. 4F) were expressed normally in *Shh*$^{-/-}$ mutant embryos, indicating that the pouches are initially formed and patterned properly despite the absence of Shh. It is important to note that since the mutants are smaller than their control littermates, direct comparisons between control and mutant embryos were not necessarily reliable indicators of expression levels. A more conservative estimate of the differences between mutants and controls was based on the relative expression in different structures within the same embryo. For example, although the expression of *Pax1* in the *Shh*$^{-/-}$ E9.5 embryo looks elevated overall, the relative levels of expression in the three pouches were roughly equivalent, similar to the pattern seen in the control littermate (Figs. 4E and F).

By E10.5–E11.0 (38 somites), both *Fgf8* and *Pax1* were abnormally expressed in *Shh* mutants. *Fgf8* expression failed to be downregulated in the second pouch of the *Shh*$^{-/-}$ mutant and appears elevated in relation to the third

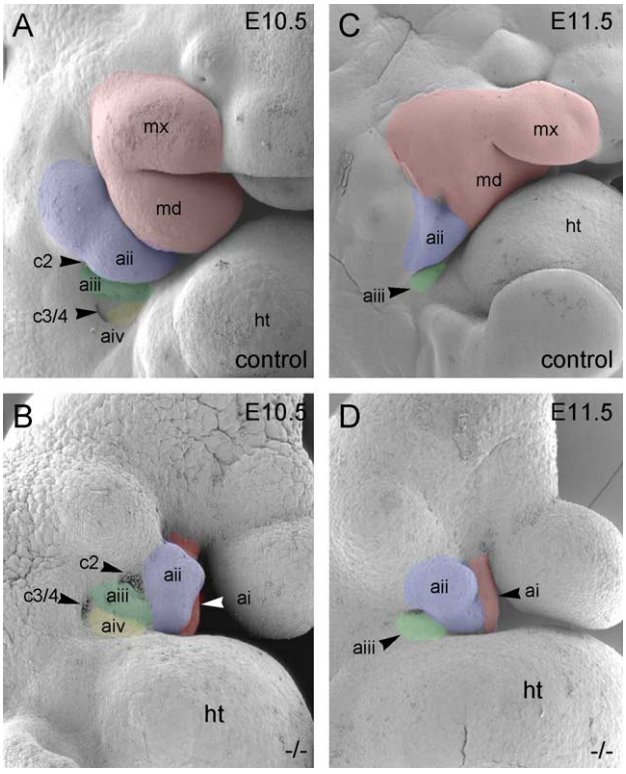

Fig. 3. Scanning electron microscopy of E10.5 (A and B) and E11.5 (C and D) *Shh*$^{-/-}$ mutants and control littermates. SEMs are pseudocolored as a visual aide and along with the arch identity markers from Fig. 2 identify the arch structures present in the *Shh*$^{-/-}$ mutant and control littermates at these stages. At E10.5, the atrophy of the more anterior arches is apparent in the mutant (B). Although the third and fourth arches are always smaller by comparison to the more anterior arches even in the control, they are still visible in the *Shh*$^{-/-}$ E10.5 mutant (A and B). By E11.5, SEM (D) shows the severely affected although remaining arch structures in Shh mutant embryos. SEMs were pseudocolored to highlight the arches; red/mx/md, maxillary/mandibular arch; blue/aii, second arch; green/aiii, third arch; yellow/aiv, fourth arch; c2–4, clefts; ht, heart.

pouch, as compared to the pattern of expression observed in the littermate control (Figs. 4C and D). In E10.5 control embryos, *Pax1* was expressed at a higher level in the endoderm of the third pouch relative to the anterior pouches (Fig. 4G). However, in the E10.5 *Shh*$^{-/-}$ mutant, *Pax1* expression remained high in the second pouch and in the pharyngeal endoderm of the presumptive remnant of the first pouch (Fig. 4H). Therefore, expression of both *Fgf8* and *Pax1* in the first and second pharyngeal pouches appears to be negatively regulated by Shh at E10.5, a change that corresponds to the appearance of *Shh* expression within the first and second pouches at this stage (Figs. 1I and J). In contrast, *Hoxb1* expression in the fourth pouch was similar in control and *Shh* mutant embryos at E10.5 (Figs. 4I and J). The relatively normal expression of *Fgf8* and *Pax1* in the third pouch and of *Hoxb1* in the fourth suggests that *Shh* is not required for maintenance of third and fourth pouch identity, consistent with its exclusion from these pouches.

Pharyngeal region morphology in the Shh mutant

The SEM analysis and *Pax1* and *Dlx3* expression patterns in *Shh* mutants showed that the first pouch and arch do initially form, but by E10.5 the first arch has atrophied and the first pouch was subsequently lost. To further confirm this result, we examined the morphology of the pharyngeal region by histological analysis and 3D reconstructions of the pharyngeal endoderm in E9.5 and E10.5 control and *Shh*$^{-/-}$ mutant embryos. Transverse sections of control and mutant embryos showed the presence of the first and second pouches at E9.5 (Figs. 5A and C). This result was confirmed with 3D reconstructions based on these sections (Figs. 5B and D). By E10.5, the first, second, third, and fourth pouches had fully formed in the control embryo (Figs. 5E and F). However, the *Shh* mutant did not have an identifiable first pouch, although they did have well-defined second, third, and fourth pouches (Figs. 5G and H, and data not shown). This result is consistent with the gene expression results showing that although the first pouch endoderm maintained some Pax1 expression, morphologically the first pouch is lost after E9.5.

Increased cell death and a decrease in cell proliferation in Shh mutants

Shh$^{-/-}$ mutants consistently exhibited reductions in both pouch and arch size, often showing a reduced cellularity in H&E-stained sections. Previous studies have shown that downregulation of Shh signaling by addition of anti-Shh antibodies (Ahlgren and Bronner-Fraser, 1999; Ahlgren et al., 2002) or NCC-specific deletion of *Smo* (Jeong et al., 2004) results in apoptosis of neural crest cells, suggesting that Shh signaling is required for NCC survival. Because changes in cellularity can reflect differences in cellular proliferation or programmed cell death, we examined these cellular processes in E9.5–E10.5 control and *Shh* mutant embryos using a cell death marker lysotracker red and a cell proliferation marker anti-phosphohistone H3. In control E9.5 embryos, we found cell death in the pharyngeal mesenchyme surrounding the first pouch, within the otic vesicle, and at very low levels in the second arch mesenchyme (Figs. 6A and C). By E10.5, cell death was present almost exclusively in the second and third pouch pharyngeal endoderm and in the arch mesenchyme immediately adjacent to the pouch endoderm in control embryos (Figs. 6E and G). In *Shh*$^{-/-}$ mutants, we observed a dramatic increase in cell death in the mesenchyme of both E9.5 (Figs. 6B and D) and E10.5 (Figs. 6F and H) embryos. The E9.5 cell death pattern was similar to that of immigrating neural crest cells whereas cell death at E10.5 was more extensive in the surrounding mesenchyme. In comparison to controls, cell death was also elevated in the endoderm of the first pouch at E9.5 (Fig. 6D). In contrast, there was far less cell death observed in the endoderm of the

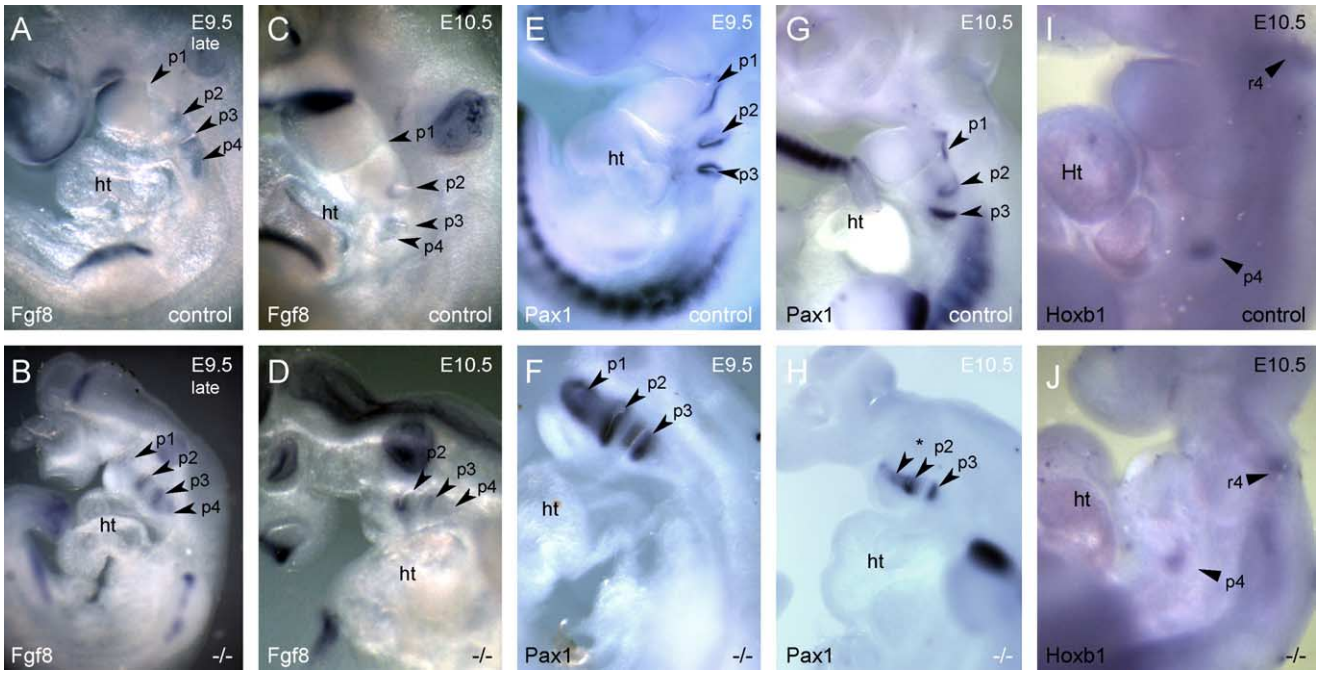

Fig. 4. Expression of pouch identity markers *Fgf8* (A–D), *Pax1* (E–H), and *Hoxb1* (I and J) in $Shh^{-/-}$ mutants and control littermates. Fgf8 is expressed in the endoderm of the second, third, and fourth pouches of the late E9.5 mutant and control embryos (A and B). At late E10.5, Fgf8 fails to be downregulated and appears elevated in the second pouch in relation to the expression observed in the third (C and D). *Pax1* expression is normal in both mutant and control embryos at E9.5 (E and F). By E10.5, *Pax1* is normally expressed predominantly in the third pouch and is downregulated in the first and second pouches (G). In E10.5 $Shh^{-/-}$ mutant, *Pax1* fails to be downregulated in the second pouch and in what appears to be the epithelial remnant of the first pouch (H). A marker for the fourth pouch, Hoxb1 is present in both control and mutant E10.5 embryos, indicating that this most posterior pouch maintains its identity (I and J). Images of the smaller $Shh^{-/-}$ mutants were taken at a higher magnification. p1–4, pharyngeal pouches; r4, rhombomere4; ht, heart.

second and third pouches in the E10.5 $Shh^{-/-}$ mutant in comparison to wild type (Fig. 6H).

Using an antibody to the phosphorylated form of histone H3, we saw similar levels of proliferating cells throughout the pharyngeal region of control (Figs. 7A and C) and mutant (Figs. 7B and D) embryos at E9.5. By E10.5, cell proliferation was reduced overall in the pharyngeal region of the control littermates, but was concentrated in the distal

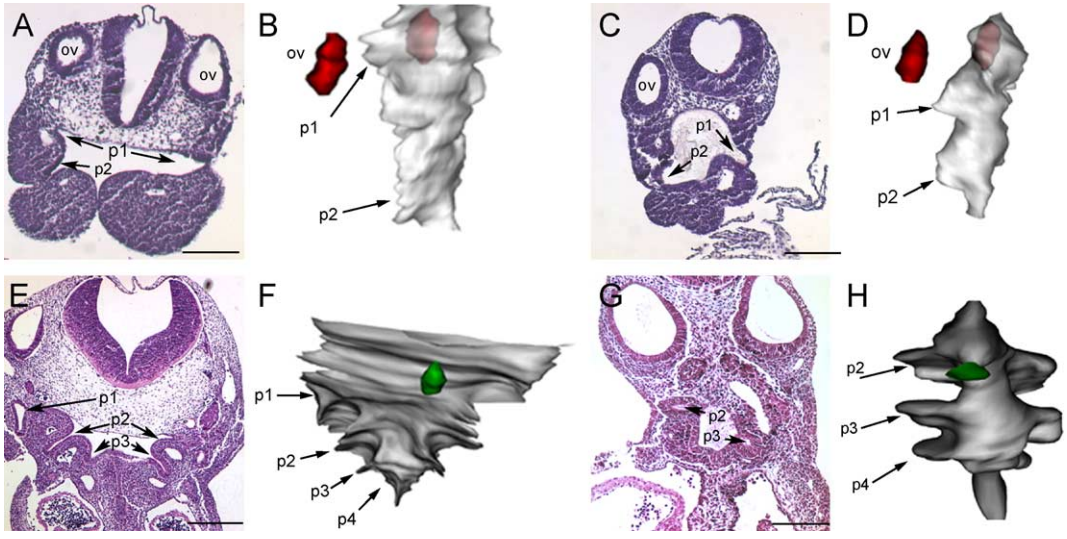

Fig. 5. E9.5 and E10.5 pharyngeal arch and pouch morphology of *Shh* control littermates and mutants. H&E sections of control and mutant embryos are displayed next to their respective 3D reconstructions. A and C are transverse sections of E9.5 (21 somites) control and $Shh^{-/-}$ mutant. E and F are coronal sections of E10.5 (37 somites) control and mutants. Note the fourth pouch is not in the plane of section used for the $Shh^{-/-}$ E10.5 mutant. The otic vesicle (red) is located dorsally between the first and second pouches and the thyroid diverticulum (green) is in the ventral pharyngeal endoderm located ventrolaterally and just above the second pouch. These structures were used for orientation purposes during the reconstructions. All reconstructions are rotated or tipped to better present the data. p1–4, pouches; scale bar, 0.3 mm.

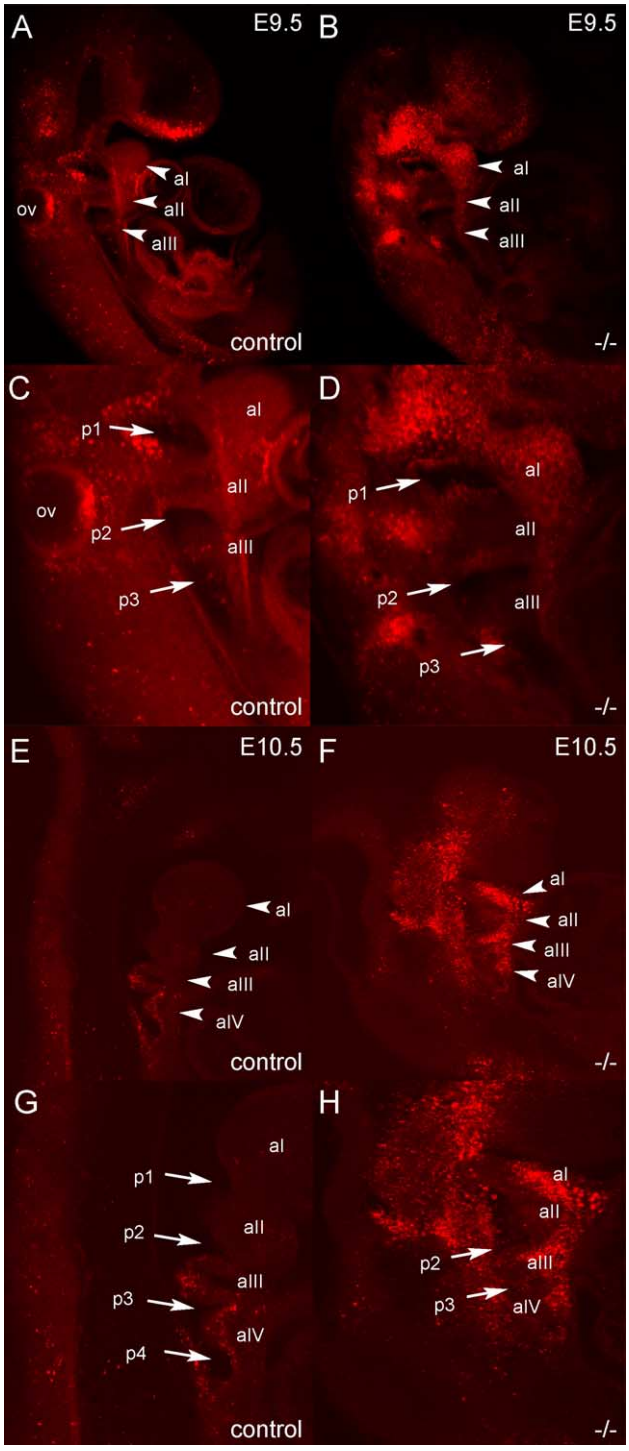

Fig. 6. CSLM analysis of cell death in whole embryos. *Shh* control littermates and mutants were examined for cell death using lysotracker red dye at both E9.5 (A, B, C, and D) and E10.5 (E, F, G, and H). A, B, E, and F are 10-μm confocal sections of embryos and C, D, G, and H are higher magnification views of the pharyngeal regions in A, B, E, and F. Cell death is evident in control embryos as a normal event in the development of pharyngeal pouches, otic vesicle, and arch mesenchyme of both E9.5 and E10.5 control embryos (A, C, E, and G). In E9.5 $Shh^{-/-}$ mutants, the cell death pattern in the pharyngeal arches is reminiscent of migrating neural crest cells (B and D). At E10.5, cell death remains elevated in arches of the $Shh^{-/-}$ mutants (F and H). aI–IV, arches; p1–4, pharyngeal pouches.

first and second arches (Fig. 7C). In mutant E10.5 embryos, there appears to be a loss of this highly proliferative zone in the distal first arch, consistent with previous reports (Jeong et al., 2004). Despite the absence of this zone of high proliferation in Shh mutants, there was no significant difference in overall pharyngeal region cell proliferation between control and mutant embryos at either stage (Fig. 7E, E9.5 control, 293 ± 23, $n = 6$; mutant 295 ± 65, $n = 6$; E10.5 control, 286 ± 18; mutant, 279 ± 28, $n = 6$). Thus the reduction in overall cellularity found in the $Shh^{-/-}$ mutant pharyngeal region and the atrophy of the first arch is primarily a consequence of an increase in cell death as opposed to decreased cell proliferation.

Shh signaling regulates Bmp4 expression and dorsal–ventral patterning of the third pouch endoderm

Bmp4 often functions in opposition to the Shh pathway in the establishment of specific domains in multiple tissues and has also been shown to regulate *Shh* expression (Watanabe et al., 1998; Zhang et al., 2000; Zhao et al., 2000). Therefore, we examined the expression of *Bmp4* in the pharyngeal region in $Shh^{-/-}$ mutant and control embryos. In control E10.5 embryos, *Bmp4* was expressed in the first and second arch ectoderm and mesenchyme, in the ventral portion of the second cleft ectoderm, the dorsal portion of the second pouch and surrounding mesenchyme, and in the ventral/posterior domain of the third pouch (Fig. 8A). In the $Shh^{-/-}$ mutants, we found that *Bmp4* expression was lost in the remnant of the first arch and from the second arch. In the second cleft and pouch *Bmp4* expression remained similar to control embryos (Fig. 8B). However, *Bmp4* expression was expanded throughout the endoderm of the third pouch. These results suggested that Shh has differential effects on *Bmp4* expression along the A/P axis, acting as a positive regulator in the first and second arches and as a negative regulator in the third pouch.

The changes in *Bmp4* expression in the third pouch in $Shh^{-/-}$ mutants at E10.5 suggested that there may be defects in dorsal–ventral patterning. The third pouch is normally patterned into dorsal parathyroid and ventral thymus-specific organ domains by E10.5 (Blackburn and Manley, 2004; Gordon et al., 2001). The thymus and parathyroid organs are derived from a shared organ primordium, which is an epithelial outgrowth of third pouch endoderm. At this stage, *Pax1* is normally expressed in the pharyngeal region only in the bilateral 3rd pouch-derived primordia and is downregulated in the other pouches (Fig. 8C). However, two bilateral *Pax1*-positive structures were present in the pharyngeal region of the E11.5 $Shh^{-/-}$ mutant (Fig. 8D). To identify these *Pax1*-positive structures, we used a marker specific to the developing thymic rudiment, *Foxn1*. *Foxn1* expression was present in both control and mutant thymic lobes (Figs. 8E and F), identifying the more posterior pair of *Pax1*-positive structures as the thymic lobes (Fig. 8D). This result is consistent with previous data

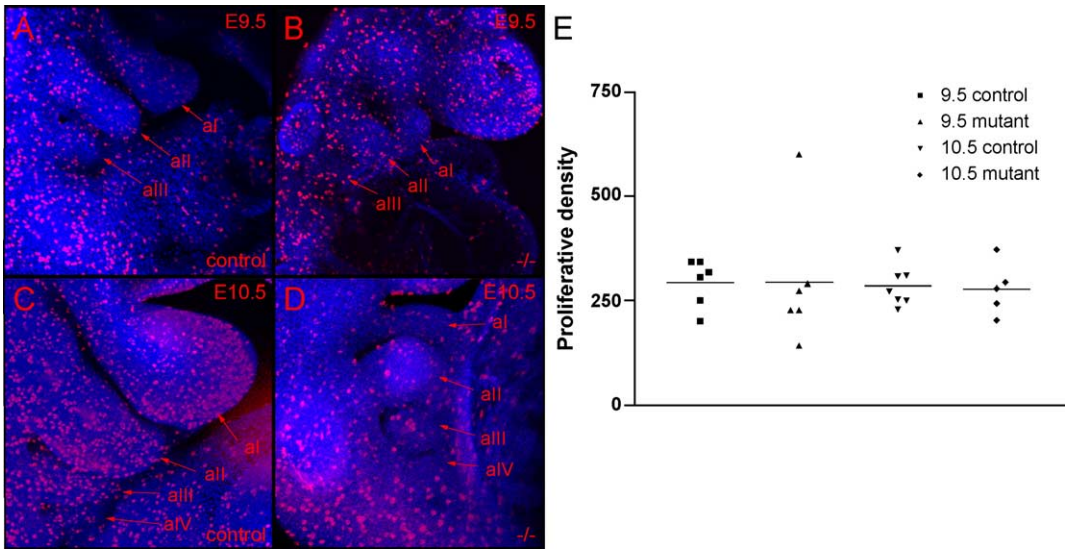

Fig. 7. Cell proliferation in control and *Shh* mutant embryos. Both E9.5 and E10.5 whole embryos were analyzed for cell proliferation in the pharyngeal region with a primary anti-phosphohistone H3 antibody detected with an Alexa red 495-conjugated secondary antibody. Cell nuclei were stained with DAPI. At E9.5, cell proliferation appeared reduced in the $Shh^{-/-}$ mutant in comparison to the control embryos (A and B). By E10.5, cell proliferation is similar between the control and $Shh^{-/-}$ mutant (C and D). However, there was no statistically significant difference in cellular proliferation between the mutants and controls (E). aI–IV, arches.

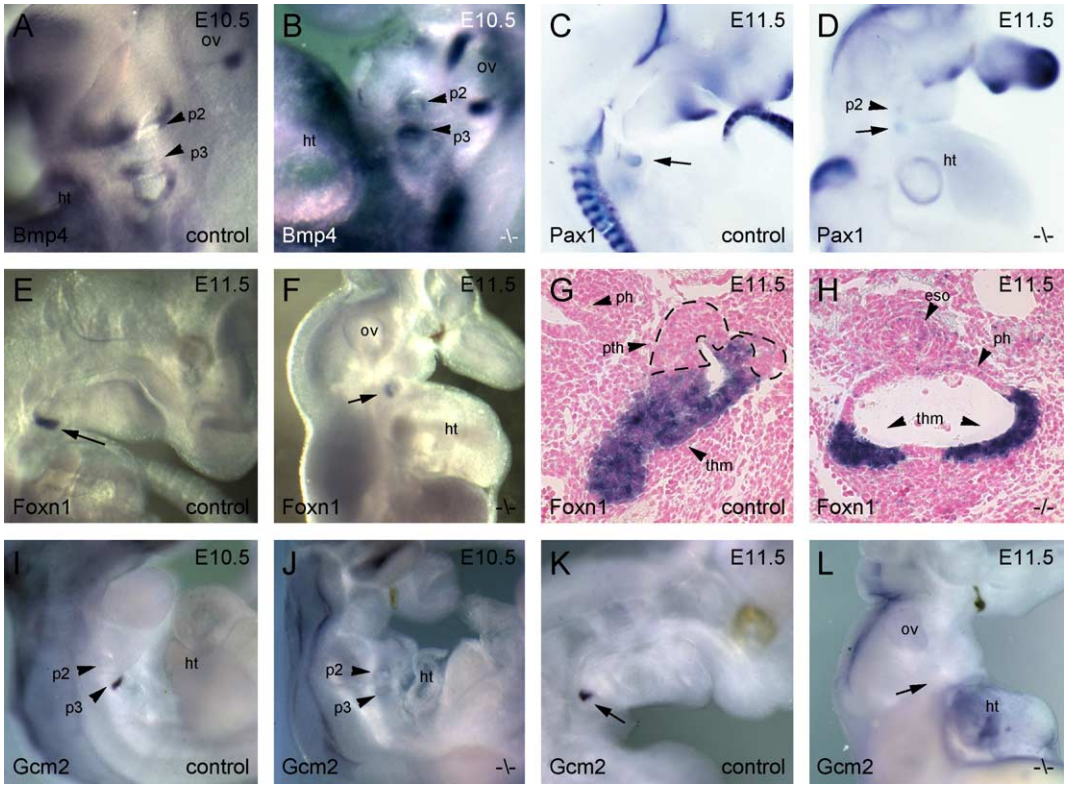

Fig. 8. Patterning of the third pouch and shared parathyroid/thymus organ primordium is disrupted in the $Shh^{-/-}$ mutant. In E11.5 controls, the proximally located parathyroid domain of the shared primordium is marked by *Gcm2* expression (A), the distal portion is the *Foxn1*-positive thymus domain (C and inset), and *Pax1* is expressed throughout the epithelial rudiment (E). In the E11.5 $Shh^{-/-}$ mutant, *Gcm2* remains undetectable (B) and *Foxn1* has expanded proximally (D). *Pax1* expression is present but is expressed in two bilateral structures in the pharyngeal region (F). The upper pair appears to be persistent second pouch structures and the lower pair is the thymic rudiment. pth, parathyroid; th, thymus; md, mandibullar; mx, maxillary; aII, second arch; aIII, third arch; p2, second pouch; ht, heart; ov, otic vesicle; ph, pharynx; fl, forelimb.

showing that the thymus does form in *Shh*$^{-/-}$ mutants, although it has a functional defect (Shah et al., 2004). The more anteriorly located pair of *Pax1*-positive structures is therefore likely to represent persistent expression of *Pax1* in the second pouch, which would have normally regressed by E11.5.

The *Pax1* and *Foxn1* expression patterns suggested that initial thymus development occurred at the normal time and place in *Shh* mutant embryos. To determine whether parathyroid development was affected, we used *Gcm2* as a parathyroid-specific marker (Gordon et al., 2001). At E10.5, Gcm2 was normally expressed in a specific dorsal and anterior presumptive parathyroid domain in control embryos (Figs. 8I and K). In contrast, *Gcm2* expression was undetectable in both E10.5 and E11.5 *Shh* mutants (Figs. 8J and L). Consistent with the loss of *Gcm2* expression, parathyroids were not identified in *Shh* mutants (data not shown). This result suggested that Shh is required for the establishment of the dorsal parathyroid-specific domain in the third pouch.

The lack of *Gcm2* expression and presence of *Foxn1* expression at E11.5 suggested that the third pouch-derived organ primordium did form, but may not have been patterned appropriately into organ-specific domains. We performed in situ hybridization for *Foxn1* on paraffin sections of E11.5 control and *Shh* mutant embryos to investigate the patterning of the primordium in more detail. While *Foxn1* expression was restricted to the ventral and distal primordium in controls, in *Shh*$^{-/-}$ mutants, *Foxn1* expression was expanded throughout the entire primordium and even into the endoderm of the pharynx itself (Figs. 8G and H). Taken together, these results suggested that the absence of *Shh* at E10.5 leads to loss of *Gcm2* expression and expansion of *Bmp4*, which results in a loss of dorsal parathyroid fate and expansion of the ventral thymus fate in the developing third pouch.

Discussion

In summary, the expression and function of *Shh* are consistent with a stronger dependence of the anterior pharyngeal structures on Shh. Shh is required for the maintenance of pharyngeal arch morphology, most likely acting as a survival factor from the endoderm on the surrounding arch mesenchymal cells. The decrease in cellularity in *Shh*$^{-/-}$ mutants, caused by an increase in cell death, and its subsequent impact on the neural crest cell population, do not disrupt arch identity. Despite its presence in the pharyngeal endoderm, Shh as previously described, does not contribute to the initial patterning and formation of the pharyngeal region, although at later stages Shh acts as a repressor of major pouch identity markers. *Shh* expression was notably excluded or undetectable in regions of the pharyngeal endoderm that are associated with organogenesis. Our results provide evidence that separate mechanisms regulate arch and pouch identity, and show that Shh signaling plays multiple roles in the developing pharyngeal region. Here we show that *Shh*, a morphogen found in many signaling centers throughout development, is differentially expressed in the pharyngeal endoderm of the mouse.

At E9.5, *Shh* is expressed throughout the ventral and dorsal pharyngeal endoderm but is downregulated in pouch endoderm. At this stage in *Shh* mutants, the arches and pouches have formed, indicating that Shh is not required for their initial formation, as previously described (Ahlgren and Bronner-Fraser, 1999; Chiang et al., 1996). However, by E10.5–E11.0, the first arch is significantly reduced in size and the first pouch is lost, and the remaining arches though present are smaller than those of the controls. This phenotype corresponds to an increase in *Shh* and *Ptc1* expression in the endoderm of the first and second pouches, suggesting that Shh signaling is stronger in the anterior arch tissues and decreases posteriorly along the pharyngeal A/P axis (Fig. 9A). This higher level of activity in the anterior portion of the pharyngeal region results in more severe structural and patterning phenotypes in the anterior first and second arches and pouches and a less severe posterior defect, with only partial loss of patterning in the third pouch and no apparent defects in the fourth.

Our results show that Shh is required for the maintenance of arch mesenchyme, as its absence results in a dramatic increase in mesenchymal cell death contributing to the mutant phenotype. In E9.5 *Shh*$^{-/-}$ mutants, the cell death pattern we observed is similar to that of migrating neural crest cells and is consistent with previous reports showing increased cell death in the presence of Shh neutralizing antibody in chick (Ahlgren and Bronner-Fraser) or in mouse embryos with an NCC-specific deletion of *Smoothened* (Jeong et al.). In spite of this high level of cell death in the arch mesenchyme, there is no effect on the initial establishment of arch and pouch patterning or on the maintenance of arch identity. This result agrees with previous findings in the chick in which the pharyngeal region was shown to pattern normally in the absence of neural crest cells (Veitch et al., 1999). Furthermore, *Shh* mutants also had higher cell death in the first pouch, but lower cell death in the posterior pouches. These results indicate that the role of Shh in endoderm survival is dynamic along the A–P axis and that Shh has multiple roles in the development of pharyngeal pouch endoderm.

There is a clear difference in the expression pattern of *Shh* in the pharyngeal region of chickens and mice. In the chick, *Shh* expression is present in the anterior endoderm of pouches 1–3 and elevated in the anterior endoderm of the second pouch (Wall and Hogan, 1995). In mice, *Shh* is initially undetectable in all pouch endoderm, then becomes expressed in the endoderm of the first and second pouches but is still undetectable in the third and fourth at E10.5 (Figs. 1 and 9). As Shh controls cell survival in both chick and mouse embryos, it would be interesting if this difference

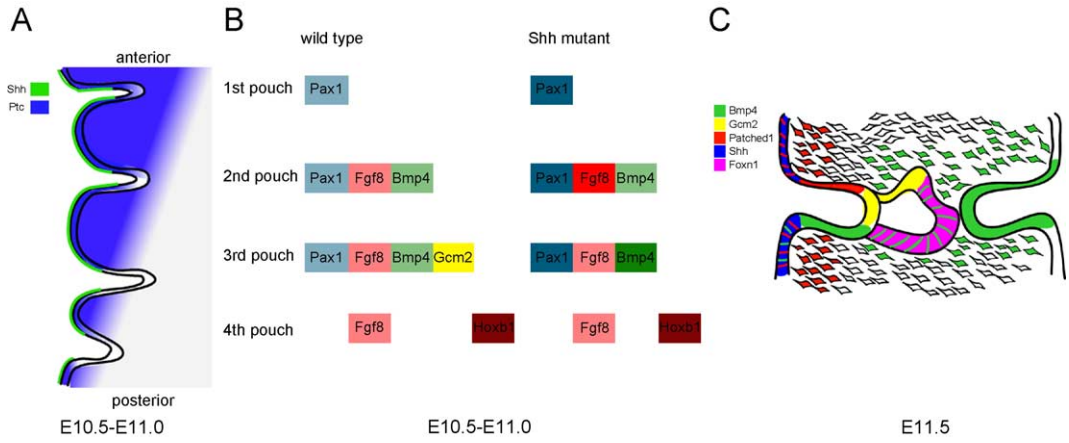

Fig. 9. Summary diagram. (A) Wild-type expression patterns of both *Shh* and *Ptc1* show that this pathway is expressed differentially from the anterior to the posterior portion of the pharyngeal region at E10.5–E11.0. (B) One role for Shh in the pharyngeal region is to repress the expression of several pouch endoderm marker genes. Different shades of color indicate the patterns of expression seen for these markers in the *Shh* mutant, with lighter to darker shades indicating lower to higher intensity of expression observed. Pax1 is normally expressed predominantly in the third pouch at E10.5, whereas in the mutant it is expressed similarly in the first, second, and third. Wild-type Fgf8 expression is similar in the second, third, and fourth pouches while in the mutant more expression is relatively higher in the second pouch. Finally, Bmp4 expression is expanded throughout the endoderm of the third pouch of the mutant while it normally is restricted to the posterior region. (C) This expansion during the initial stages of organogenesis in the third pouch later impacts on the development of the shared thymic/parathyroid primordium. Our current model suggests that Shh and Bmp4 regulate the patterning of the primordium into the Gcm2 (parathyroid specific) and Foxn1 (thymus specific) domains, and that Shh represses *Bmp4* expression in the anterior domain of the third pouch. In the absence of Shh, *Bmp4* expression expands along with the *Foxn1*-positive thymus domain of the primordium.

in expression contributes to a difference in arch morphology or subsequent organ development between these species.

Exclusion of *Shh* expression has been shown to be associated with the emergence of organ primordia, including the dorsal pancreatic bud and Rathke's pouch, and misexpression of *Shh* perturbs pancreas and pituitary formation. However, *Shh* expression is necessary at later stages in pancreas and pituitary development, contributing to the differentiation of specialized cell types within each organ (Hebrok, 2003; Hebrok et al., 2000; Sbrogna et al., 2003; Treier et al., 2001). Our data indicate that this same mechanism is acting within the pharyngeal endoderm in the genesis of multiple organs. The data are most striking in the third and fourth pouches, where both Shh and Ptc1 expressions are low or undetectable in the domains that will form the thymus and ultimobranchial bodies, respectively. In contrast, in the dorsal-anterior parathyroid domain within the third pouch, *Shh* is excluded, but *Ptc1* is upregulated, indicating that in the third pouch, Shh signaling is required for parathyroid, but not thymus organogenesis. This conclusion is supported by the loss of parathyroid identity and organ formation and expansion of thymus formation in the *Shh* mutants. During later fetal development, Shh is also required for normal thymus development and function after initial organ formation (Shah et al., 2004) (Moore-Scott, unpublished data).

We have shown that at E10.5–E11.0, a time point important for the initial stages of organogenesis and remodeling of the pharyngeal region, $Shh^{-/-}$ mutants develop abnormalities in the patterning of the second and third pouches as shown by misexpression of *Pax1*, *Fgf8*, *Gcm2*, and *Bmp4* (Fig. 9B). The expression of *Pax1* and *Fgf8*, which are elevated in the second pouch, and *Bmp4*, which expands in the third pouch in the $Shh^{-/-}$ mutants, indicates that Shh normally represses these genes at this stage. Furthermore, the absence of *Gcm2* expression in the dorsal anterior portion of the third pouch and the expansion of *Foxn1* in $Shh^{-/-}$ mutants show that Shh is required for dorsal–ventral regionalization of the third pouch endoderm. The expansion of *Bmp4* in the third pouch suggests a mechanism by which opposing *Shh* and *Bmp4* signals establish dorsal/ventral polarity of the third pouch and subsequent organ primordium (Fig. 9C). Thus, loss of Shh results in absence of dorsal parathyroid identity and expansion of ventral thymus fates within the primordium. These data are also consistent with previous studies suggesting that Bmp4 is a positive regulator of Foxn1 expression in the fetal thymus (Tsai et al., 2003), and suggest that Bmp4 may also play a role in initial induction of *Foxn1* expression.

Our results indicate that regulation of pouch identity by Shh is either downstream or independent of arch identity, since A–P patterning of the arches is apparently unaffected in $Shh^{-/-}$ mutants. This is somewhat different from the posterior endoderm, where misexpression of *Shh* in the chick induced ectopic *Hoxd11* and *Hoxd13* in the early stages (HH8–13) of hindgut development, suggesting that in the more posterior endoderm, Shh is upstream of *Hox* gene expression (Roberts et al., 1998). Although *Hoxa3* gene expression was not affected in *Shh* mutants, the third pouch-derived organ phenotype is in some ways reminiscent of the *Hoxa3* knockout phenotype, which fails to initiate formation of the thymus/parathyroid primordium (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995) and does not

express Gcm2 (S. Ellis and NR Manley, unpublished data). This similar phenotype raises the possibility that *Shh* is downstream of *Hoxa3,* at least in parathyroid development. However, *Shh* expression was unaltered in *Hoxa3*$^{-/-}$ null mutants (Moore-Scott, unpublished data), suggesting that *Shh* and *Hoxa3* do not regulate each other's expression. Thus, *Hoxa3* and *Shh* appear to be independently required for *Gcm2* expression and parathyroid organogenesis. Furthermore, although *Shh* expression has not been reported in *Hoxa2*$^{-/-}$ mutants, *Fgf8* expression is unaffected in *Hoxa2* mutants (Bobola et al., 2003), but is changed in *Shh* mutants. As the other *Hox2* and *Hox3* paralogous genes are not expressed in the pharyngeal endoderm, *Shh* expression in the pharyngeal endoderm is unlikely to be dependent on Hox gene expression. Since we found no evidence that either Shh regulates the expression of Hox genes in this region or that these genes directly regulate Shh expression, it is unclear as to how Shh is regulated in what is clearly a positionally dependent fashion. It is possible that multiple paralogous Hox genes could regulate *Shh* expression in a combinatorial manner or indirectly through induction of regulatory factors in the surrounding mesenchyme. Recent work has indicated that the cumulative total of Hoxd genes present within different regions of the limb could affect the ability of Gli3 to act as repressor or activator (Chen et al., 2004). Therefore, *Hox* genes could regulate the expression or activity of downstream effectors of the hedgehog pathway in this region thereby contributing to differential hedgehog activity in an AP restricted manner.

Acknowledgments

Many thanks to Julie Gordon, Scott Dougan, and Scott Stadler for helpful discussions concerning the manuscript, and to Sammy Navarre for assistance with mouse husbandry. Thanks to Ellen Ritchie and Monica Zamisch for technical assistance with paraffin section in situ hybridization. Multiphoton and confocal scanning laser microscopy were performed at the Center for Ultrastructural Research at the University of Georgia. Thanks to Mark Farmer and John Shields for assistance with the confocal analysis. This work was supported by grants from the National Institutes of Health to N.R.M. (HD35920 and HD043479).

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