The concentric structure of the developing gut is regulated by Sonic hedgehog derived from endodermal epithelium

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SUMMARY

The embryonic gut of vertebrates consists of endodermal epithelium, surrounding mesenchyme derived from splanchnic mesoderm and enteric neuronal components derived from neural crest cells. During gut organogenesis, the mesenchyme differentiates into distinct concentric layers around the endodermal epithelium forming the lamina propria, muscularis mucosae, submucosa and lamina muscularis (the smooth muscle layer). The smooth muscle layer and enteric plexus are formed at the outermost part of the gut, always some distance away from the epithelium. How this topographical organization of gut mesenchyme is established is largely unknown. Here we show the following: (1) Endodermal epithelium inhibits differentiation of smooth muscle and enteric neurons in adjacent mesenchyme. (2) Endodermal epithelium activates expression of *patched* and *BMP4* in adjacent non-smooth muscle mesenchyme, which later differentiates into the lamina propria and submucosa. (3) *Sonic hedgehog* (*Shh*) is expressed in endodermal epithelium and disruption of Shh-signaling by cyclopamine induces differentiation of smooth muscle and a large number of neurons even in the area adjacent to epithelium. (4) Shh can mimic the effect of endodermal epithelium on the concentric stratification of the gut. Taken together, these data suggest that endoderm-derived Shh is responsible for the patterning across the radial axis of the gut through induction of inner components and inhibition of outer components, such as smooth muscle and enteric neurons.

Key words: Epithelial-mesenchymal interaction, Shh, Patched, BMP4, cFKBP/SMAP, SCG10, Smooth muscle, Enteric neuronal cell, Radial axis

INTRODUCTION

The embryonic gut of vertebrates consists of endodermal epithelium, mesenchyme from splanchnic mesoderm and neuronal components. Although the gut is initially a simple tube, it becomes divided anteroposteriorly into various organs such as the esophagus, stomach (proventriculus and gizzard in the chicken), duodenum, small intestine and large intestine. Differentiation along the anterior-posterior (AP) axis has been extensively studied and the importance of epithelial-mesenchymal interactions in organogenesis is now well established (reviewed in Yasugi, 1993; Kedinger et al., 1988).

In contrast to the considerable variation of histological architectures along the AP axis, organization of the gut across the radial axis is essentially similar at any level of the AP axis. During organogenesis of the digestive organs in the chicken embryo, as in other vertebrates, the mesenchyme segregates into four distinct concentric layers, depending on the distance from the epithelium. The innermost layer of the mesenchyme just adjacent to the epithelium differentiates into the lamina propria, the next layer gives rise to the muscularis mucosae,

outside this the submucosa differentiates, and the outermost region forms the circular and longitudinal smooth muscle layers (Calhoun, 1954; Romanoff, 1960). Precursors of the enteric nervous system derived from neural crest cells (Yntema and Hammond, 1954, 1955; Le Douarin and Teillet, 1973) enter the gut at the level of the pharynx (Le Douarin and Teillet, 1973; Tucker et al., 1986) and migrate caudally through the outer part of the gut mesenchyme where they proliferate, and ultimately differentiate into neurons and glia. These cells form two kinds of neural plexus, which control motility and secretions of the gut (Allan and Newgreen, 1980; Fairman et al., 1995); one is situated at the outer part of submucosa and the other between the layers of muscle. There have been few studies of the regulatory mechanisms underlying this topographically organized differentiation of the gut, but we have previously shown that chicken gut smooth muscle cells express cFKBP/SMAP, a member of the FK-506 binding protein family (Fukuda et al., 1998).

Shh has been shown to have crucial roles in patterning of a diverse range of tissues (reviewed in Hammerschmidt et al., 1997). For example, Shh expressed in the posterior limb

mesenchyme is required for establishment of its AP axis (Riddle et al., 1993; Laufer et al., 1994). Shh secreted from notochord and floor plate is necessary for the determination of the dorso-ventral axis of the neural plate by the induction of ventral phenotypes such as floor plate and motorneurons (Tsuchida et al., 1994; Ericson et al., 1995, 1997a,b; Roelink et al., 1994, 1995; Tanabe and Jessell, 1996). In somites, Shh from the notochord and the floor plate of the neural tube acts as a positive signal for differentiation of myotome (Munsterberg et al., 1995; Stern et al., 1995), whereas BMP4 produced in lateral mesoderm negatively regulates myogenic differentiation (Pourquie et al., 1996; Tonegawa et al., 1997). In developing gut, it has been shown that *Shh* is expressed in the endodermal epithelium and induces *patched* and *BMP-4* expression in mesenchyme of gut (Roberts et al., 1995, 1998; Marigo et al., 1996; Narita et al., 1998).

In this study, we have investigated the expression and function of the genes mentioned above to elucidate the molecular mechanisms involved in concentric differentiation of chicken gut mesenchyme. We found that all gut mesenchymal cells have the potency to differentiate into smooth muscle cells and that epithelium inhibits this differentiation. We also showed that epithelium inhibits proliferation of enteric neurons and controls their distribution within the gut mesenchyme. Furthermore, Shh could mimic the effect of epithelium on the topographical differentiation of gut. Disruption of Shh signaling with a steroidal alkaloid, cyclopamine, resulted in induction of smooth muscle and a large number of the enteric neuronal cells in mesenchyme adjacent to the epithelium. We further demonstrated that *BMP4* was expressed in the inner non-muscle mesenchymal layer and that virus-derived BMP4 inhibited enteric neuronal differentiation but did not disturb smooth muscle differentiation. Taken together, we conclude that epithelial-mesenchymal interaction is required for the mesenchymal stratification across the radial axis in the gut and that Shh-BMP4 signaling is important for this interaction.

MATERIALS AND METHODS

In situ hybridization and immunohistochemistry

In situ hybridization with digoxigenin-labeled probes was performed on 12 μm cryosections as described by Ishii et al. (1997). cDNA fragments of *Shh* (Nohno et al., 1995), *patched* (a gift from Dr C. J. Tabin; Marigo et al., 1996), chick *BMP4* (Francis et al., 1994), mouse *BMP4* (a gift from Dr K. Shibuya; Kurihara et al., 1993), *cFKBP/SMAP* (Fukuda et al., 1998), *SCG10* (Stein et al., 1988), *Xenopus noggin* (a gift from Dr Y. Sasai) were used as probes. For immunohistochemistry, 12 μm cryosections were treated with monoclonal antibody 13F4 (a gift of Dr N. M. Le Douarin; Rong et al., 1987) or QCPN (Selleck and Bronner-Fraser, 1995), which recognizes a muscle-specific antigen and quail cell-specific antigen, respectively. Antibody binding was visualized using Cy3-conjugated donkey anti-mouse IgM (Jackson ImmunoResearch Lab. Inc.) or HRP-conjugated goat anti-mouse IgG (Cappel) and DAB, respectively.

Organ culture and epithelial-mesenchymal recombination

Gizzard and small intestine were isolated from 6-day embryonic chicken. Epithelium and mesenchyme were separated by collagenase treatment as described in Ishii et al. (1997) and Narita et al. (1998). During the isolation and subsequent procedures, special attention was paid to keep identifying inner (epithelial) and outer (mesenterial) sides of the mesenchyme. Epithelia were recombined on the inner side (type I), outer side (type II), or both sides of mesenchyme (type III). In one

type of culture the mesenchyme was cultured without epithelium (type IV) (see Fig. 1). These recombinants were cultured in vitro for 4 days in medium 199 (Gibco-BRL) containing 50% embryo extract as described by Takiguchi et al. (1988) and Fukuda et al. (1998).

Shh-expressing cell line and organ culture

A Shh-expressing cell line was established from quail QT6 cell (QT6-Shh, a gift from Dr Y. Aoyama; Watanabe et al., 1998). Isolated gizzard mesenchyme from 6-day embryo was placed on a porous membrane (Nuclepore SN11049, 8 μm pore size; Costar). Cells were transplanted onto the outer surface of the mesenchyme and tissue-cell recombinants were cultured for 4 days (see Fig. 6A).

Cyclopamine treatment

10 mM cyclopamine (a gift of Dr W. Gaffield; Gaffield and Keelar, 1996; Incardona et al., 1998) in 95% ethanol was diluted with medium 199 to a final concentration of 0.1-5 μM. The culture medium for cyclopamine treatment did not contain embryo extract. As a control, an equivalent amount of ethanol was added to the culture medium.

Virus-mediated overexpression of Shh and BMP4

For efficient viral infection to mesenchyme, we used embryonic tissues from pathogen-free White Leghorn C/O strain (Nippon Institute for Biological Science). Full-length cDNAs of mouse *BMP4* (a gift from Dr K. Shibuya), chicken *Shh* (a gift from Dr A. Kuroiwa) and *Xenopus noggin* (a gift from Dr Y. Sasai) were inserted into a replication-competent retroviral vector RCAS or pDS, respectively (RCAS-BMP4, pDS-Shh and pDS-Nog; Murakami et al., 1997; Kameda et al., 1999). Infection was carried out as described by Kameda et al. (1999) and Narita et al. (2000).

RESULTS

The effect of endodermal epithelium on gut smooth muscle differentiation

To elucidate a possible involvement of epithelium in topographical differentiation in gut mesenchyme, we performed experiments in which epithelium and mesenchyme of the gizzard from 6-day old embryos were recombined in vitro. Four types of recombination were carried out, as shown in Fig. 1. Type I: epithelium was reattached in its original position (inner
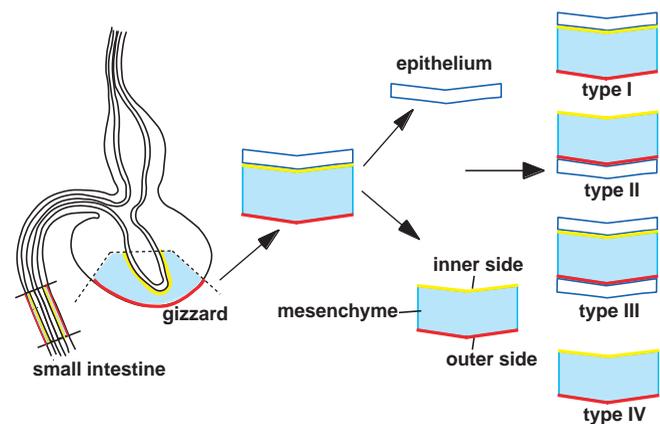

Fig. 1. Scheme of epithelial-mesenchymal recombination experiments. 6-day embryonic gizzard and small intestine were separated into epithelium and mesenchyme. Epithelia were attached to the inner (yellow) side of the mesenchymes (type I), outer (red) side of the mesenchymes (type II) or both sides of the mesenchymes (type III) and recombinants were cultured. In type IV, mesenchymes were cultured alone (see Materials and Methods).

side of the mesenchyme). Type II: epithelium was attached to the outer side of the mesenchyme. Type III: epithelium was attached to both sides. Type IV: the mesenchyme was cultured without epithelium (Fig. 1). After 4 days of culture, we analyzed the expression of *cFKBP/SMAP*, a marker of smooth muscle (Fukuda et al., 1998) and staining of 13F4, an early muscle marker, in adjacent sections. In type I recombinations outer mesenchymal cells were *cFKBP/SMAP*- and 13F4-positive (Fig. 2A,B) as in the normal gizzard (Fig. 3G), while in type II recombinations *cFKBP/SMAP* expression and 13F4-immunoreactivity were detected in the region that normally does not differentiate into smooth muscle (Fig. 2C,D). In type III recombinations *cFKBP/SMAP* and 13F4 were expressed only in the central part of the mesenchyme (Fig. 2E,F). Thus, in all types of recombinations, *cFKBP/SMAP*-positive smooth muscle cells were located at some distance away from the epithelium. Finally, in type IV recombinations, almost all mesenchymal cells expressed *cFKBP/SMAP* and 13F4 (Fig. 2G,H). These results clearly indicate that mesenchyme has an intrinsic potency to differentiate into smooth muscle cells, and that smooth muscle differentiation in mesenchyme is negatively regulated by epithelium.

Expression patterns of Shh-regulated molecules, cFKBP/SMAP and SCG10 in the gut

Since expression of *Shh* has been reported in gut epithelium (Roberts et al., 1995, 1998; Marigo et al., 1996; Narita et al., 1998), we decided to examine the effect of Shh as a candidate for an epithelium-derived negative regulator of smooth muscle differentiation. We also examined the expression of *patched* and *BMP4*, known to be activated by Shh in a variety of tissues (reviewed in Hammerschmidt et al., 1997). Expression of *cFKBP/SMAP* and *SCG10* (Stein et al., 1988) were used as smooth muscle and pan-neuronal marker, respectively. On days 6 and 9 of incubation, *Shh* expression was restricted to epithelium in all regions of gut as reported by Roberts et al. (1995, 1998) and Narita et al. (1998) (Fig. 3A,F,K,P and data not shown). A gradient of *patched* expression was observed with highest levels in cells adjacent to the epithelium, suggesting that this expression is induced by Shh from the epithelium (Fig. 3B,G,L,Q). *BMP4* was expressed in the mesenchyme just beneath the epithelium (Fig. 3C,H,M,S). The mesenchymal area just outside the *BMP4*-expressing area expressed *cFKBP/SMAP* (Fig. 3D,I,N,S), indicating the differentiation of smooth muscle.

In developing gut, enteric neuronal cells expressing *SCG10* appeared in 6-day embryonic stomach (Fig. 3E,J) and were found in progressively more caudal regions of the gut (Fig. 3O,T). These cells were situated at the outermost part of the gut. In 9-day gut, the expression patterns of these genes were similar to, but clearer than those of 6-day gut (Fig. 3F-J,P-T).

Thus, *patched* and *BMP4* were expressed in non-muscle mesenchyme, which differentiates into the

submucosa and lamina propria, but not in the smooth muscle layer.

The effect of epithelium on the expression of mesenchymal genes

To confirm the possibility that epithelium is involved not only in smooth muscle differentiation, but also in establishment of mesenchymal concentric stratification, we performed tissue recombination experiments as described in Fig. 1 and analyzed the expression pattern of mesenchymal genes described above in serial sections of explants after 4 days of culture. In type I, II and III recombinations, *Shh* was expressed only in the epithelium (Fig. 4A,F,K), both *patched* and *BMP4* were expressed in the mesenchyme adjacent to the epithelium (Fig. 4B,C,G,H,L,M), and *cFKBP/SMAP* in the region just outside the *BMP4*-expressing layer (Fig. 4D,I,N). *SCG10*-expressing enteric neuronal cells were always located on the opposite side to the epithelium (Fig. 4E,J). There were very few enteric
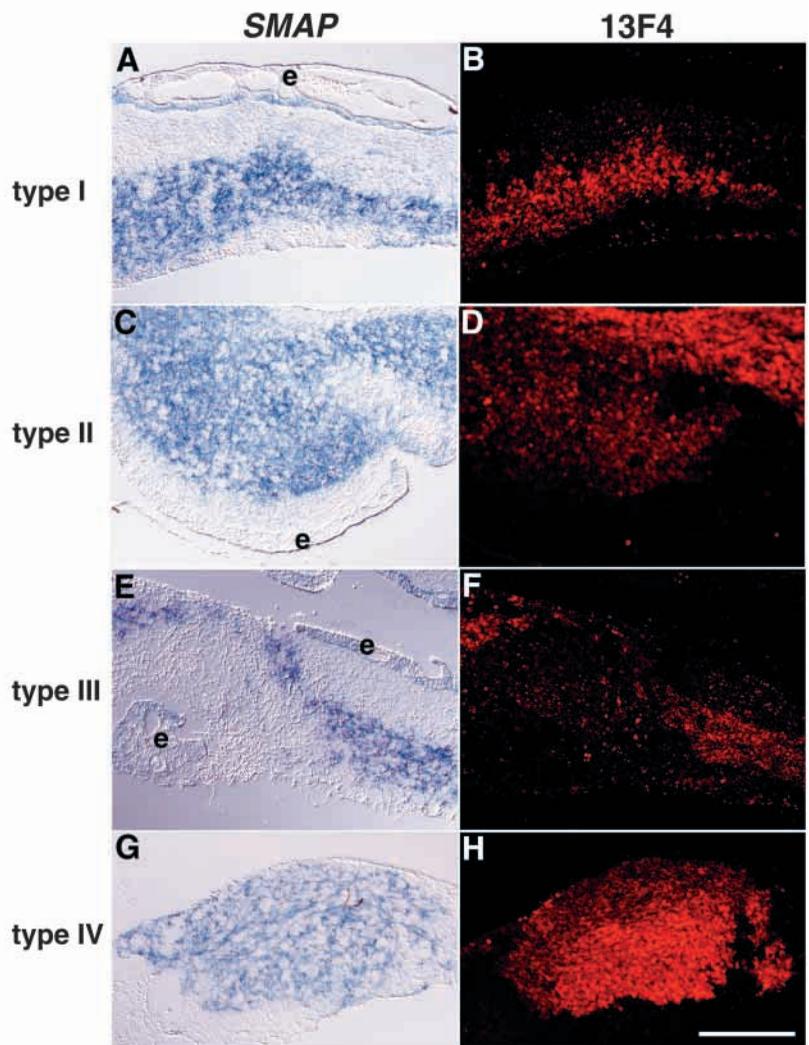

Fig. 2. Effect of epithelium on the smooth muscle differentiation in gizzard mesenchymal explants. The expression of *cFKBP/SMAP* (*SMAP*; A,C,E,G) and 13F4 immunoreactivity (13F4; B,D,F,H) in serial sections of type I (A,B), type II (C,D), type III (E,F) and type IV (G,H) explants were analyzed by in situ hybridization and immunohistochemistry. e, epithelium. Bar, 200 μm.
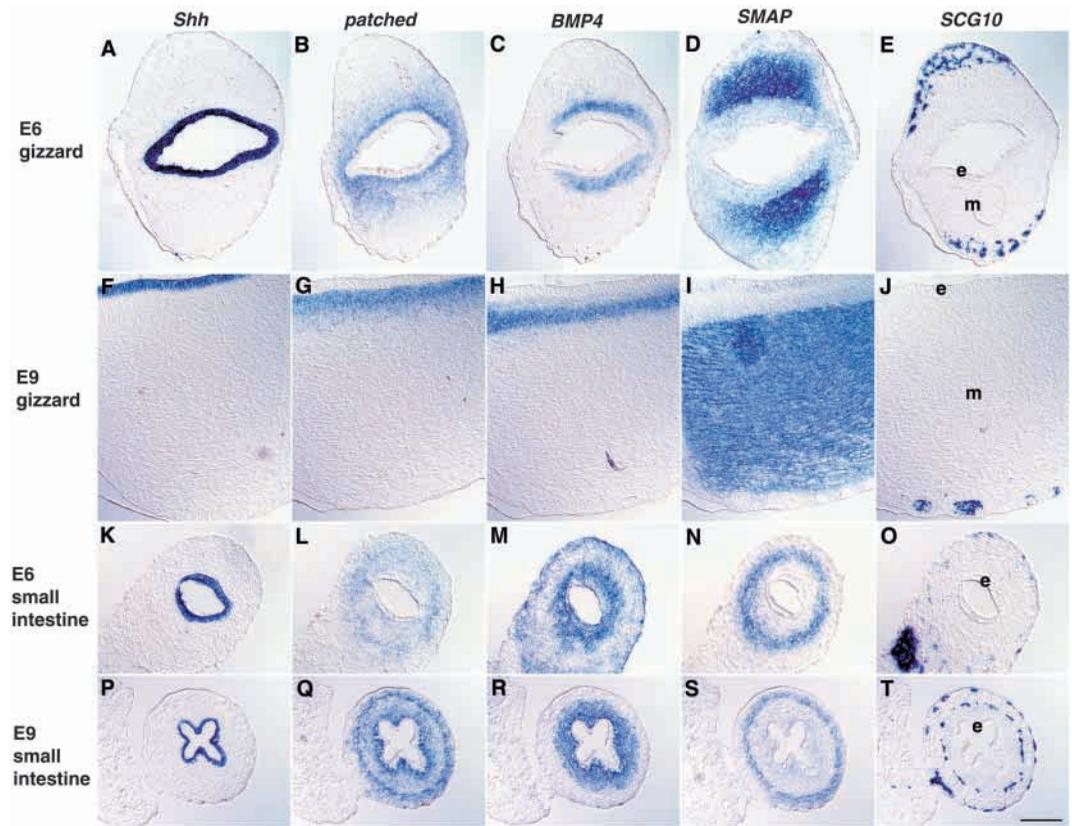
Fig. 3. The expression patterns of *Shh* (A,F,K,P), *patched* (B,G,L,Q), *BMP4* (C,H,M,R), *cFKBP/SMAP* (*SMAP*; D,I,N,S) and *SCC10* (E,J,O,T) were analyzed in serial sections of 6.5-day embryonic gizzard (A-E) and small intestine (K-O), and 9-day embryonic gizzard (F-J) and small intestine (P-T), by in situ hybridization. e, epithelium; m, mesenchyme. Bar, 200 μm (A-J, P-T); 400 μm (K-O).
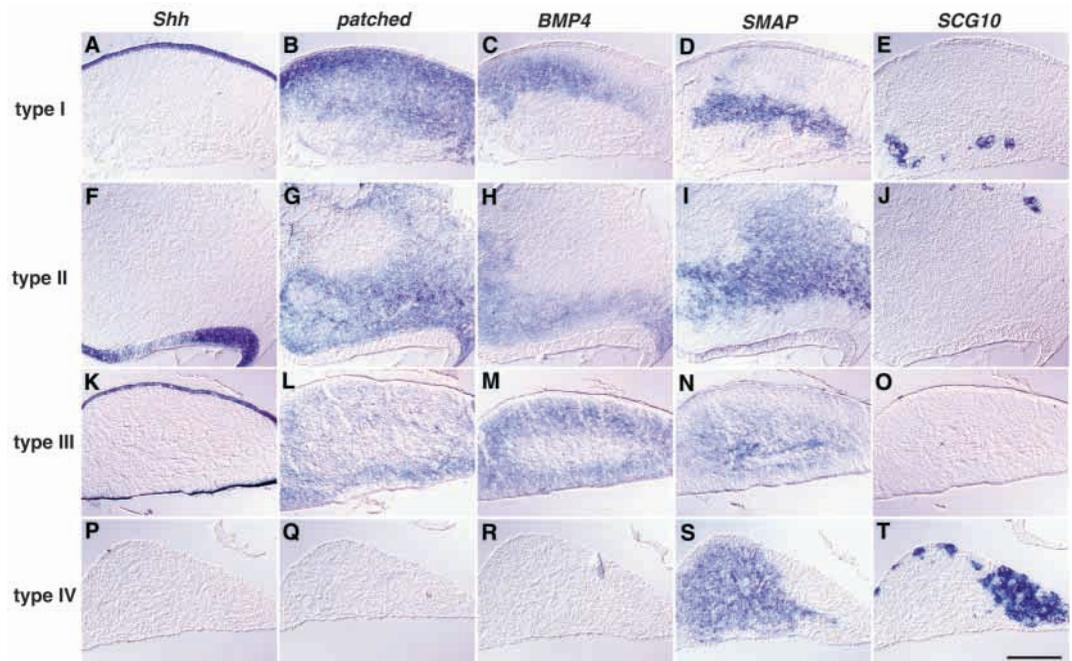

Fig. 4. Effect of epithelium on the mesenchymal stratification of gizzard explants. The expression patterns of *Shh* (A,F,K,P), *patched* (B,G,L,Q), *BMP4* (C,H,M,R), *cFKBP/SMAP* (*SMAP*; D,I,N,S) and *SCC10* (E,J,O,T) in serial sections of type I (A-E), type II (F-J), type III (L-O) and type IV (P-T) explants were analyzed by in situ hybridization. Bar, 200 μm.

neuronal cells in the central part of type III explants (Fig. 4O). In mesenchyme cultured without epithelium (type IV), expression of *Shh*, *patched* and *BMP4* was not detected (Fig. 4P-R); most mesenchymal cells expressed *cFKBP/SMAP* (Fig. 4S) and a large number of enteric neuronal cells differentiated (Fig. 4T).

These results indicate that epithelium induces both *patched* and *BMP4* expression in neighboring non-smooth-muscle mesenchyme of the gut, and that epithelium also inhibits the differentiation of the enteric neuronal cells.

Disruption of Shh activity by cyclopamine induces smooth muscle differentiation

In the following experiments, we analyzed epithelial factors that

Fig. 5. Effect of disruption of Shh activity by cyclopamine on the mesenchymal stratification of gizzard explants. The expression of *Shh* (A,F,K,P), *patched* (B,G,L,Q), *BMP4* (C,H,M,R), *cFKBP/SMAP* (*SMAP*; D,I,N,S) and *SCC10* (E,J,O,T) in serial sections of explants cultured with 0 μM (A-E), 0.1 μM (F-J), 1 μM (L-O) and 5 μM (P-T) of cyclopamine were analyzed by in situ hybridization. Bar, 200 μm.
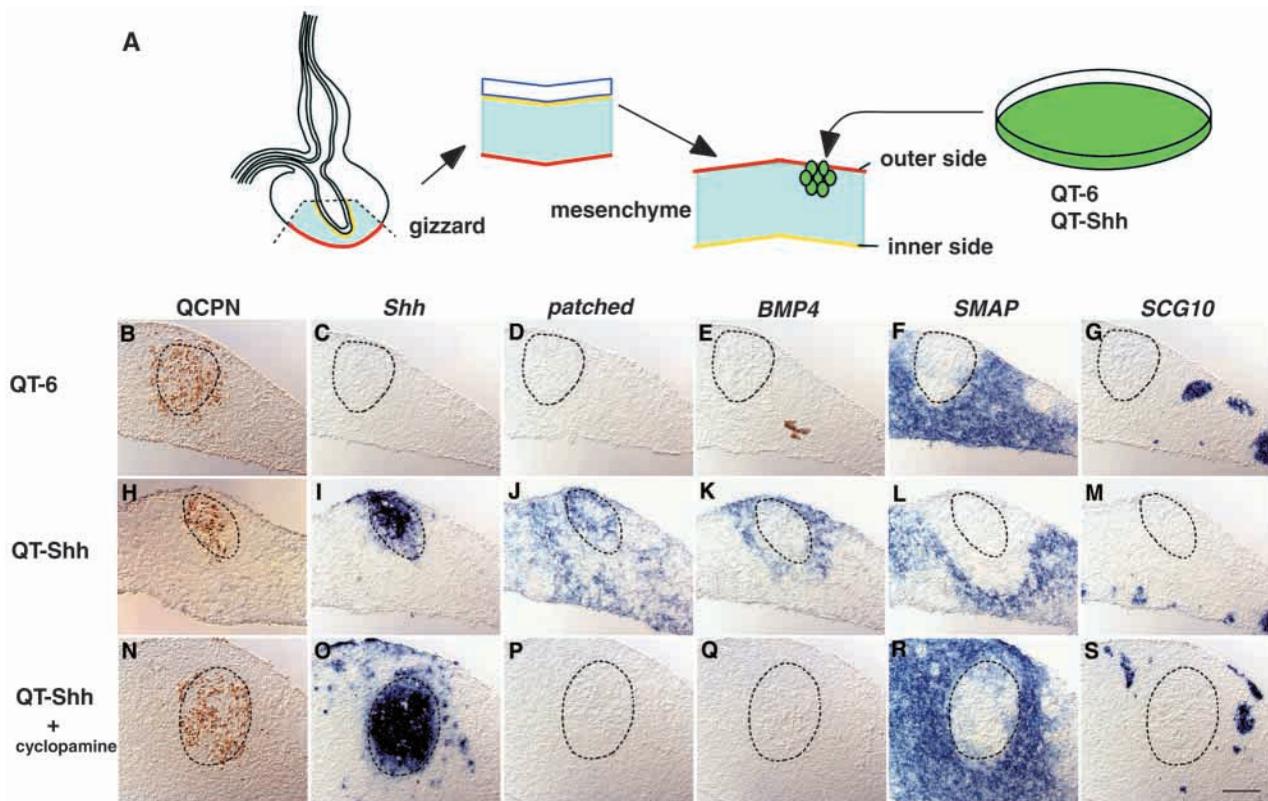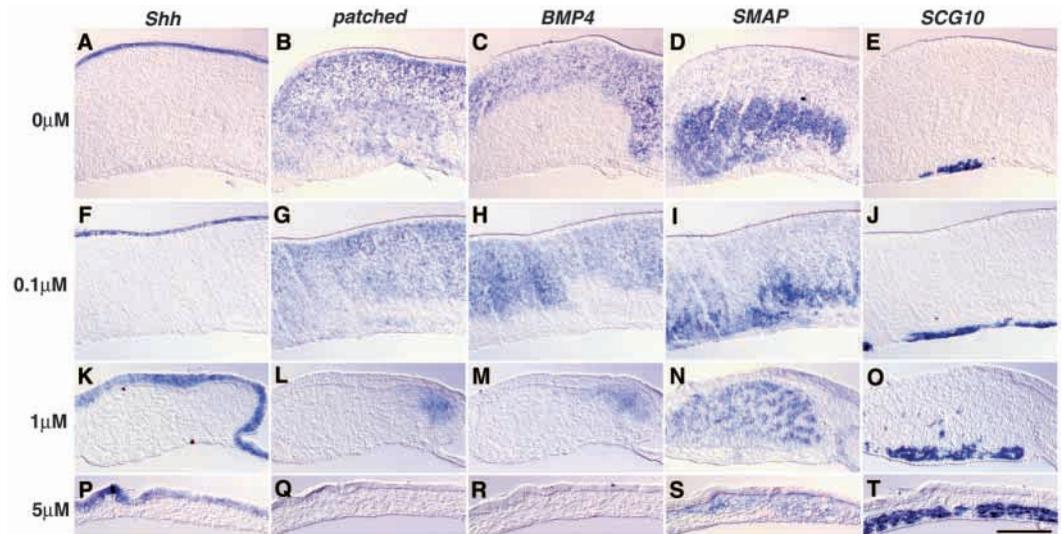
Fig. 6. Effect of Shh-expressing cells on the mesenchymal stratification of gizzard explants. The experimental procedure is shown in (A). QT-6 cells (QT-6; B-G) or Shh-expressing QT-6 cells (QT-Shh; H-S) were transplanted on the outer surface of 6-day embryonic gizzard after removal of epithelium. QT6-Shh-transplanted explants were cultured without (H-M) or with 5 μM cyclopamine (QT-Shh+cyclopamine; N-S). The expression of *Shh* (C,I,O), *patched* (D,J,P), *BMP4* (E,K,Q), *cFKBP/SMAP* (*SMAP*; F,L,R) and *SCG10* (G,M,S) were analyzed by in situ hybridization and quail cells were detected by immunoreactivity to monoclonal antibody QCPN (QCPN: B,H,N) in serial sections. Hatched line circles indicate areas of implanted quail cells. Bar, 400 μm.

might mediate the regulation of concentric structure in the gut. The expression of *Shh* in gut epithelium and the observation that the epithelium induced *BMP4* expression in adjacent mesenchyme (Roberts et al., 1995; Fig. 4) implicate Shh as a potential signal from epithelium to mesenchyme. We therefore examined the function of epithelium-derived Shh on the

differentiation of mesenchyme using the steroidal alkaloid, cyclopamine (Keeler, 1969), which has been shown to block Shh-mediated patterning of the neural tube (Porter et al., 1996; Incardona et al., 1998) and endoderm (Kim and Melton, 1998). The expression of *Shh*, *patched*, *BMP4*, *cFKBP/SMAP* and *SCG10* was analyzed in gizzard cultured with 0.1-5 μM
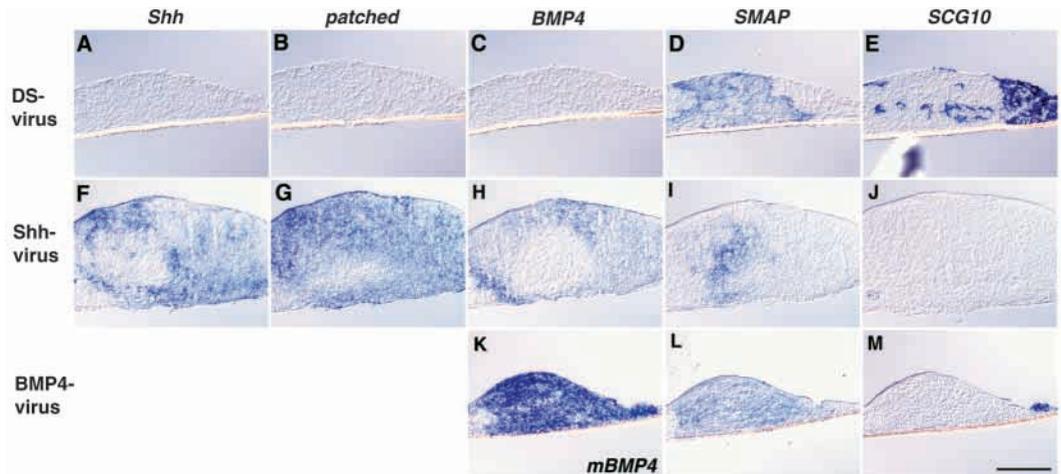
Fig. 7. Effect of virus-mediated Shh and BMP4 on the mesenchymal stratification of gizzard explants. The gizzard mesenchymes were cultured after infection by control virus, pDS (DS-virus; A-E), Shh-expressing virus, pDS-Shh (Shh-virus; F-J) or BMP4-expressing virus, RCAS-BMP4 (BMP4-virus; K-M). The expression patterns of *Shh* (A,F), *patched* (B,G), *BMP4* (C,H), mouse *BMP4* (m*BMP4*; K), *cFKBP/SMAP* (*SMAP*; D,I,L) and *SCG10* (E,J,M) were analyzed by in situ hybridization in serial sections. Bar, 200 µm.

cyclopamine for 4 days. There was no significant change of gene expression patterns in the explants treated with no or low doses (0.1-0.5 µM) of cyclopamine (Fig. 5F-J), while in explants cultured with a high dose (1-5 µM) of cyclopamine, the regions of *patched*- and *BMP4*-expressing mesenchymal cell were much reduced (Fig. 5K-T). The number of enteric neuronal cells increased, depending on the concentration of the cyclopamine (Fig. 5E,J,O,T). In explants cultured with 5 µM cyclopamine all mesenchymal cells expressed either *cFKBP/SMAP* (Fig. 5S) or *SCG10* (Fig. 5T). A large number of enteric neuronal cells were observed in the mesenchyme adjacent to the epithelium (Fig. 5T), despite the maintenance of *Shh* expression in the epithelium (Fig. 5P). It was noted that cyclopamine-treated explants were smaller than control ones, suggesting that hedgehog signaling may also be involved in the growth of mesenchyme, as previously described (Kim and Melton, 1998). These results demonstrate that Shh derived from epithelium is required for the topographical patterning of gut mesenchyme.

Shh can mimic the effect of the epithelium

We further tested whether Shh alone could mimic the effect of epithelium on mesenchymal differentiation. We transplanted two quail cell lines, QT6 cells or Shh-expressing QT6 cells (QT-Shh) onto the outer surface of isolated 6-day embryonic gizzard mesenchyme after removal of the epithelium (Fig. 6A) and cultured the explants for 4 days. Only implanted QT-Shh cells, which were detected by the quail cell marker, QCPN (Fig. 6H,N), expressed *Shh* (Fig. 6I,O), since mesenchymal cells do not express endogenous *Shh* (see above). Both *patched* and *BMP4* expressions were induced in the mesenchymal cells adjacent to the QT-Shh cells (Fig. 6J,K). In contrast, *cFKBP/SMAP* expression was absent only in this area (Fig. 6L). *SCG10*-positive enteric neuronal cells were always located far from QT6-shh cells (Fig. 6M). Parental QT6 cells (Fig. 6B) did not induce expression of *patched* or *BMP-4* (Fig. 6D,E), and *cFKBP/SMAP*-positive smooth muscle cells (Fig. 6F) and *SCG10*-positive neuronal cells (Fig. 6G) were observed. When QT-Shh-transplanted explants were cultured with 5 µM cyclopamine, the expression patterns of *patched*, *BMP-4*, *cFKBP/SMAP* and *SCG10* (Fig. 6P-S) were similar to those in QT6-transplanted explants.

Misexpression of *Shh* in mesenchyme after removal of epithelium (Fig. 7F) by Shh-virus also induced high expression of *patched* and *BMP4* (Fig. 7G,H), as shown in the previous report (Roberts et al., 1998), and suppressed expression of *cFKBP/SMAP* and *SCG10* (Fig. 7I,J) in the area where *Shh* was expressed. These results demonstrate that Shh can mimic the activity of gut epithelium to regulate topographical patterning of gut mesenchyme.

The effect of BMP4 on the differentiation of smooth muscle and enteric neuronal cells

In this study, we have shown that *BMP4* expression is complementary to *cFKBP/SMAP* expression in gut mesenchyme (Fig. 3). In order to test the possibility that the effect of Shh on the establishment of concentric structure in the gut was mediated by BMP4, virus-mediated overexpression of mouse BMP4 in mesenchyme without epithelium was carried out (Fig. 7K). Overexpression of BMP4 did not affect *cFKBP/SMAP* expression in mesenchyme (Fig. 7L), while the number of neuronal cells was much reduced (Fig. 7M). In addition, while there was no effect on *cFKBP/SMAP* expression in explants in which *Xenopus noggin* was misexpressed using retrovirus to inhibit BMP signaling in mesenchyme (Fig. 8G-K), *SCG10*-positive neuronal cells appeared ectopically in BMP4-expressing mesenchyme in this type of explants (Fig. 8L). Taken together, these results suggest that BMP4 mediates the role of epithelium on differentiation of enteric neuronal cells, but is not involved in smooth muscle differentiation.

DISCUSSION

Shh is a endodermal factor that regulate mesenchymal differentiation

Shh secreted from epithelium is important in a number of morphogenetic events during gut development. Shh expression is first detected in the endodermal epithelium just after the establishment of the digestive tube (Bitgood and McMahon, 1995; Narita et al., 1998) and continues throughout development (Narita et al., 1998). During early development
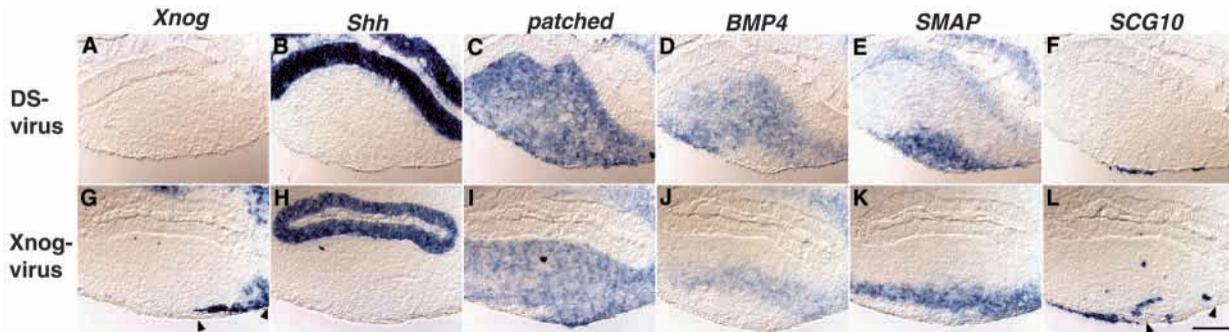
Fig. 8. Effect of inhibition of BMP activity by virus-mediated noggin on the mesenchymal stratification of gizzard explants. Gizzard was cultured after infection by control virus, pDS (DS-virus; A-F) or *Xenopus* noggin-expressing virus, pDS-noggin (Xnog-virus; G-L). The expression patterns of *Xenopus noggin* (*Xnog*; A,G), *Shh* (B,H), *patched* (C,I), *BMP4* (D,J), *cFKBP/SMAP* (*SMAP*; E,K) and *SCG10* (F,L) were analyzed by in situ hybridization in serial sections of explants. Noggin-expressing mesenchyme is situated between the arrowheads in G and L. Bar, 400 µm.

of the mouse, Shh is required both for maintenance of epithelium and for its own expression in endoderm (Chiang et al., 1996; Litingtung et al., 1998; Motoyama et al., 1998). Later in development Shh is involved in the region-specific differentiation of both epithelium and mesenchyme. For example, in the presumptive pancreatic epithelium, downregulation of Shh induces the expression of pancreas-specific genes (Hebrok et al., 1998; Kim and Melton, 1998) and the pancreatic mesenchyme is converted into intestinal mesenchyme by ectopic Shh expression in pancreatic epithelium (Apelqvist et al., 1997). We have also found that Shh regulates gene expression in the gland epithelium of the proventriculus (our unpublished results).

In this study, we have demonstrated a new function for Shh: patterning across the radial axis of the gut (Fig. 9). All gut mesenchymal cells appear to have the potency to differentiate into smooth muscle cells. However, Shh-mediated signaling inhibits differentiation of smooth muscle, resulting in differentiation of non-muscle layers such as the lamina propria and submucosa. The direct influence of epithelium-derived Shh on the mesenchyme can be monitored by the expression of Shh-responsive genes such as *patched* and *BMP4* (Roberts et al., 1998). Expression patterns of these genes demonstrate that the influence of Shh is limited to cells in close proximity to the epithelium, the source of Shh. We have shown that cFKBP/SMAP is necessary and sufficient for smooth muscle differentiation in gut (Fukuda et al., 1998). Therefore, it is possible that smooth muscle cell differentiation is inhibited near the epithelium as a consequence of inhibition of *cFKBP/SMAP*. Shh secreted from the epithelium also inhibited the differentiation of enteric neuronal cells, which consequently are restricted to regions distant from the source of Shh. The experiments described were carried out using day-6 gizzard and small intestine, in which the concentric patterning is already established (Fig. 3). The mechanism described above may therefore be involved in maintenance and/or repatterning of the mesenchyme. However, in mesenchymes taken from 4-day embryonic gizzard and small intestine, which do not yet express *BMP4* and *cFKBP/SMAP*, smooth muscle was induced in mesenchyme at some distance away from QT6-Shh cells (data not shown), suggesting that initial patterning occurs by a similar mechanism.

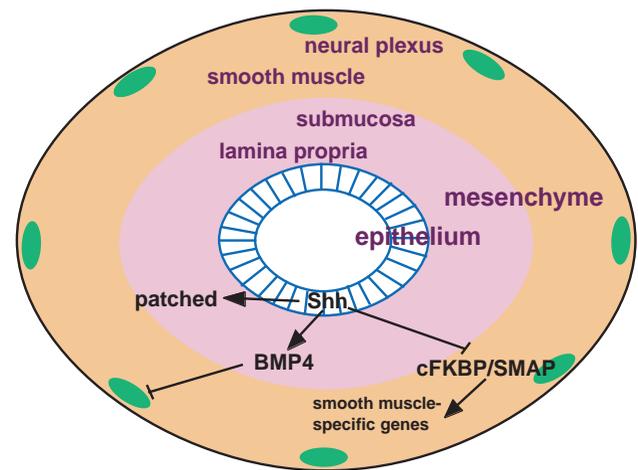In the mouse, *Shh* is similarly expressed in gut epithelium

Fig. 9. Schematic summary of the molecular mechanism of gut differentiation across its radial axis. Shh from epithelium induces *patched* and *BMP4* expression in adjacent non-smooth muscle mesenchyme, which will differentiate into lamina propria and/or submucosa. Shh also inhibits expression of *cFKBP/SMAP*, a regulatory molecule of smooth muscle differentiation, in mesenchyme near the epithelium. BMP4 induced by Shh from epithelium inhibits enteric neuronal differentiation. Thus, smooth muscle and enteric neuronal cells differentiate in the outer region of the gut (see Discussion).

(Bitgood and McMahon, 1995). Analysis of enteric neuronal differentiation in *Shh*-knockout mice is consistent with our results (Dr M. R. Santos, personal communication). In such mice there is an increase in the number of enteric neuronal cells and their distribution within the mesenchyme is abnormal. Also, smooth muscle always differentiates some distance away from epithelium in the gut of mice. However, the epithelium is necessary for smooth muscle differentiation in the mouse stomach (Takahashi et al., 1998) and intestine (Kedinger et al., 1990). Ectopic Shh in pancreatic epithelium induces smooth muscle differentiation in pancreatic mesenchyme (Apelqvist et al., 1997). Although these reports may appear to conflict with our data, it is possible that Shh may initially act as an inducer and later as an inhibitor of smooth muscle differentiation. In mesenchyme from 4-day embryonic chicken gizzard smooth muscle did not differentiate without epithelium (our unpublished data), supporting this idea.

Another member of hedgehog, *Indian hedgehog* (*Ihh*), was co-expressed with *Shh* throughout gut epithelium (Matsumoto et al., 1998). *Ihh* induced *patched* and *BMPs* in vertebrate cartilage (Vortkamp et al., 1996). Overexpression of *Ihh* in gut mesenchyme also induced the expression of *patched* and *BMP4*, and inhibits the expression of *cFKBP/SMAP* and *SCG10* (our unpublished data). This data suggests that *Shh* and *Ihh* in endodermal epithelium affect mesenchymal patterning cooperatively.

The function of BMP4 on the differentiation of gut mesenchyme

In the wing disc of *Drosophila* (Kojima et al., 1994; Tabata and Kornberg, 1994; Capdevila and Guerrero, 1994; Tabata et al., 1995; Zecca et al., 1995), vertebrate cartilage (Kim et al., 1998; Murtaugh et al., 1999), tooth (Dassule and McMahon, 1998; Tucker et al., 1998) and limb (Kawakami et al., 1996; Zou et al., 1997; Merino et al., 1998), members of the hedgehog family have been shown to act through induction of TGFβs (reviewed by Hammerschmidt et al., 1997). In this report we have demonstrated that BMP4, the TGFβ family member, is involved in gut mesenchymal patterning. BMP4 did not affect *cFKBP/SMAP* expression but inhibited *SCG10* expression. Shh may therefore regulate enteric neuronal differentiation through induction of BMP4, while it controls smooth muscle differentiation directly or via induction of factor(s) other than BMP4 (Fig. 9).

Recently, Roberts et al. (1998) found that BMP4 was expressed throughout the chicken gut mesenchyme except in the stomach (proventriculus and gizzard) region, and that epithelial Shh induced *BMP4* expression in midgut and hindgut (small and large intestinal) mesenchyme but not in stomach mesenchyme. We show that *BMP4* is expressed throughout the gut mesenchyme (Fig. 3) and epithelial Shh induced *BMP4* expression not only in small intestinal but also in stomach (gizzard) mesenchyme (data not shown and Fig. 4). We suggest the difference between these results is due to the developmental stage of the embryo examined. Robert et al. (1998) showed the expression pattern of *BMP4* in 4-day embryos, in which the regionalization of the gut is not yet significant. Therefore, it is possible that BMP4 signaling is involved in establishment of the AP axis at early development, and is required for radial axis formation in later stages.

Enteric neuronal differentiation in gut mesenchyme

In this study we have shown that epithelium affects the distribution and differentiation of enteric neuronal cells. Enteric neuronal cells always exist adjacent to or between smooth muscle layers (Gabella, 1994; our results), where they innervate smooth muscles and regulate the gut motility. Neurturin, a neurotrophic factor that is expressed in smooth muscle cells, controls growth of the enteric neurons (Rossi et al., 1999; Heuckeroth et al., 1999). It is therefore possible that Shh determines the amount and distribution of smooth muscles which, in turn, controls enteric neuron differentiation through neurotrophic factors. However, in explants treated with a high concentration of cyclopamine, the number of neuronal cells was much increased (Fig. 5T), while there was less smooth muscle than in control explants (Fig. 5S). In mesenchyme in which *Noggin* was overexpressed, there were enteric neuronal cells in the area that did not express *cFKBP/SMAP* (Fig. 7P,Q).

In addition, FK506 inhibited differentiation of smooth muscle (Fukuda et al., 1998) but did not affect the differentiation of enteric neuronal cells (our unpublished data). These data suggest that there is no direct correlation between smooth muscle and enteric neuronal differentiation in early stages.

At later stages of development there are two layers of neuronal cells in small intestine (Fig. 3T); one is located between the submucosa and smooth muscle layer (Meissner's plexsus), another in the outer part of the smooth muscle layer (Auerbach's plexsus). The mechanism of neuronal cell differentiation in the two layers remains unknown. It is possible that the basic concentric pattern of neuronal cells as shown in Fig. 3E,O is established by epithelial Shh and, when smooth muscle differentiation becomes evident, neuronal differentiation is affected by differentiated smooth muscle cells.

In 6-day embryonic gizzard some neural crest cells have already differentiated into neuronal cells and located in the outermost layer of the mesenchyme (Fig. 3). In the experiments in which epithelium was recombined to the outer side of the mesenchyme, neuronal cells were located to the opposite side of their original position at the end of culture (Fig. 4J). *SCG10*-expressing neuronal crest cells were found exiting gradually away from transplanted QT-Shh cells, depending on the culture period of the explants (data not shown). These results suggest that differentiated neuronal cells moved because of a repulsive effect of epithelial Shh. However, when the Shh level was high there were few or no enteric neuronal cells in the explants (Figs 4J, 7J). It is possible that neuronal cells could not survive in the absence of neurotrophic factors, or that they disappeared by apoptotic effect of BMP4. The observation of Wakamatsu et al. (1998) that neurogenic crest cells survive only when they move to a correct region where the necessary neurotorophic factors are provided supports this model.

Epithelial-mesenchymal interaction in mesenchymal patterning

There have been many studies of epithelial-mesenchymal interactions in epithelial patterning and differentiation (Yasugi, 1993; Gittes et al., 1996; Kispert et al., 1996; Ohmichi et al., 1998; Wolpert, 1998), whereas only a few reports have shown that epithelial-mesenchymal interactions regulate mesenchymal patterning. In the limb, dorso-ventral patterning of mesenchyme is regulated by Wnt-7a from dorsal epithelium (Parr and McMahon, 1995), and FGFs from the AER are required for proximal-distal patterning (Wang and Sassoon, 1995; Vogel et al., 1995). In this study we have demonstrated that endodermal epithelium acts as a center for patterning of both mesenchymal and neuronal components across the radial axis of the gut, by induction of inner tissues such as lamina propria and submucosa and by suppression of differentiation of outer tissues such as smooth muscles and enteric neurons, and that these actions of the epithelium can be ascribed to Shh produced by the endodermal epithelium.

Chicken gut is subdivided into regions along the AP axis. Although the basic organization of the mesenchyme is similar in these regions, we observed differences in the relative thickness of the smooth muscle and non-muscle layers. The regional differences of mesenchymal layers are also observed in cultured organ explants (Fig. 4, and data not shown). In recent years, region-specific expression of several regulatory genes in gut

mesenchyme such as *Hox* (Yokouchi et al., 1995; Roberts et al., 1998), *Nkx2.3* (Buchberger et al., 1996) and *Barx1* (Tessier-Seta et al., 1995) have been noticed. It is possible that these genes interact with Shh signaling across the radial axis and regulate the thickness of each mesenchymal component.

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