Epithelial-mesenchymal signaling during the regionalization of the chick gut

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SUMMARY

The development of the vertebrate gut requires signaling between the endoderm and mesoderm for establishing its normal anteroposterior (AP) axis and for tissue-specific differentiation. Factors implicated in positional specification of the AP regions of the gut include endodermally expressed Sonic hedgehog (Shh), mesodermally expressed Bmp4 and members of the Hox gene family. We have investigated the roles of these factors during AP regional specification of the chick embryonic gut. Early in gut development, the endoderm sends inductive signals to the mesoderm. Shh has been implicated as one of these signals. We find a differential response to exposure of the inductive influence of Shh along the AP axis of the gut. Virally mediated misexpression of Shh results in ectopic upregulation of its receptor Ptc and a cellular proliferation throughout the gut mesoderm. Although ectopic Shh can induce Bmp4 in the mesoderm of the midgut and hindgut, Bmp4 is not induced in the stomach region of the foregut. The stomach region has a thinner layer of mesoderm than the rest of the gut suggesting that the normal function of Bmp4 could be to limit mesodermal growth in the non-stomach regions of the gut. Ectopic Bmp4 expression in the stomach results in a reduction of the mesodermal component consistent with this hypothesis. In addition to the regional restriction on Bmp4 induction, Shh can only induce Hoxd-13 in the mesoderm of the hindgut. These findings suggest that a prepattern exists in the primitive gut mesoderm prior to expression of Shh in the endoderm. The gut mesoderm is subsequently responsible for inducing region-specific differentiation of its overlying endoderm. We tested the role of Hoxd-13, normally restricted in its mesodermal expression to the most posterior region of the hindgut (cloaca), in controlling adjacent endodermal differentiation. When virally mediated Hoxd-13 is misexpressed in the primitive midgut mesoderm, there is a transformation of the endoderm to the morphology and mucin content of the hindgut. Thus, the positionally restricted expression of a Hox gene in the gut mesoderm influences the inductive signaling that leads to regionally specific differentiation of gut endoderm.

Key words: Shh, Bmp4, Hoxd13, Chick, Gut development, Epithelial-mesenchymal interaction

INTRODUCTION

The vertebrate embryonic gut tube gives rise to many of the visceral organs of the adult animal; however, the regulation of gut patterning remains poorly understood. On a morphological level, the formation of the embryonic gut along the anteroposterior (AP) axis of the embryo occurs similarly in all animals and essentially identically in all vertebrates (Gilbert, 1994). The endodermal layer begins to invaginate at two sites, first an anterior ventral invagination (the anterior intestinal portal, AIP) followed shortly by a posterior ventral invagination (the caudal intestinal portal, CIP), forming two open ended tubes. As the tubes form, splanchnic mesoderm is recruited to surround the invaginating endoderm. The gut tubes grow and extend from both the cranial and caudal embryonic ends until they meet and fuse at the yolk stalk. This primitive gut tube is initially regionalized into three broad domains along its AP axis; the foregut, midgut and hindgut. Ultimately, the foregut will give rise to the esophagus (and crop in birds), stomach (including the gizzard and proventriculus in birds) and derivative organs – thyroid, lungs, pancreas, and liver; the midgut forms the small intestine, and the hindgut forms the ceca (in birds) or appendix (in mammals), the large intestine and the cloaca (in birds) or rectum (in mammals).

Each of the AP regions of the gut is characterized by unique mesodermal and endodermal patterns of differentiation, which can be discerned by both gross morphology and histology. For example, the endoderm of the small intestine contains numerous cell types arranged in morphological structures termed villi that function in digestion and absorption and the mesoderm of the small intestine contains two thin layers of
smooth muscle, which use peristalsis to move the luminal contents.

The regionalization of the gut along the AP axis into distinct zones and the coordination of the tissue layers such that the proper endodermal tissue types are uniquely associated with the proper mesodermal tissues, depends upon extensive signaling between the endoderm and mesoderm. Early in gut development as the gut tubes form from the AIP and CIP, the endodermal signals to the splanchnic mesoderm, recruiting it to the gut tube and inducing it to undergo gut-specific differentiation to form visceral mesoderm (Kedinger et al., 1986; Roberts et al., 1995). Later, endodermally derived signals are also, in part, responsible for specifying the region-specific differentiation of the adjacent mesoderm. For example, the differentiation of the mesoderm of the midgut into smooth muscle is dependent on signals from the adjacent endoderm (Aufderheide and Ekblom, 1988; Kedinger et al., 1990). Once the mesodermal type is specified, signals then derived from the mesoderm subsequently pattern the morphological differentiation of its overlying endoderm (Haffen et al., 1983, 1987; Kedinger et al., 1986, 1988; Yasugi, 1993). For example, when the foregut mesoderm is combined with midgut endoderm, the mesoderm respecifies the endoderm to take on foregut endoderm morphology (Kedinger et al., 1986). This mesodermal regulation of endodermal phenotypes has been shown using foregut and midgut combinations in chick (Yasugi, 1993), rat (Fukamachi and Takayama, 1980) and mouse (Fukamachi et al., 1979). Such experiments have also demonstrated that there is a developmental time window in which the primitive endoderm is responsive to influence from heterologous mesoderm. The chick gut endoderm is determined, such that it differentiates autonomously to its proper region-specific morphology, between embryonic day 3.5 (E3.5) and 6 (Fukamachi and Takayama, 1980; Ishizuya-Oka, 1983; Sumiya, 1976). However, the endoderm can still be influenced to alter its differentiation pathway by heterotypic mesodermal up to E9 (Hayashi et al., 1988; Ishizuya-Oka, 1983; Takiguchi et al., 1986).

**Sonic hedgehog (Shh)** is an important signal implicated in the first phase of signaling from the endoderm to the mesoderm. **Shh** is expressed in the definitive endoderm of the gut from the time the AIP and CIP first form (Roberts et al., 1995). As the gut tube forms and undergoes morphogenesis, **Shh**’s expression domain expands and is maintained throughout the rostral-caudal extent of the gut endoderm (Bitgood and McMahon, 1995; Marigo et al., 1995; Roberts et al., 1995), overlapping partially with a highly related factor, **Indian hedgehog**, in regions within the foregut (Bitgood and McMahon, 1995). The target of the endodermal **hedgehog** signals is the adjacent mesoderm, as demonstrated by strong specific expression of the target gene patched (Ptc) throughout the visceral mesoderm (Marigo et al., 1996). **Ptc** is a universal target of hedgehog signaling, thus while its expression can indicate the tissues responding to hedgehogs during development, it does not provide insight into the particular local function of that signal. A target gene that provides more information in this regard is **Bmp4. Bmp4** itself encodes a secreted factor, of the TGFβ superfamily, which is expressed in the visceral mesoderm during early gut formation, abutting the expression of **Shh** across the tissue layers (Roberts et al., 1995). Misexpression of **Shh** in the primitive gut leads to the ectopic induction of **Bmp4** in the gut mesoderm, suggesting that it is a normal target of endodermally derived **hedgehog** signaling (Roberts et al., 1995). **Bmp4**, in turn, may act as a secondary signal downstream of **Shh** in the recruitment of visceral mesoderm to the forming gut, as the ventral mesoderm fails to close in mice homozygous for a targeted deletion in the **Bmp4** gene (Winnier et al., 1995).

Later in development, however, during the formation and branching of the lungs, **Shh** and **Bmp4** appear to have independent roles. In terms of their spatial expression pattern, the two factors are similar in their arrangement to that seen in the early gut tube, with **Shh** in the endoderm and **Bmp4** in the adjacent mesoderm. However, overexpression of **Shh** in the lung of transgenic mice produces massive overproliferation of the mesenchymal tissues of the lungs, but **Bmp4** is not induced (Bellusci et al., 1996). Moreover, **Bmp4** overexpression in the lung buds causes decreased rather than increased proliferation of cells (Bellusci et al., 1997). Whether this difference in the interactions between **Shh** and **Bmp4** reflects a stage-specific difference or reflects an underlying regionalization of the mesoderm in its response to those factors remains unclear.

In addition to its potential role in recruiting the visceral mesoderm, **Shh** also functions in establishing differential fates of the gut-derived tissues. For example, the pancreatic buds form at specific locations of the foregut where **Shh** is not expressed in the dorsal and ventral endoderm (ApeLvist et al., 1997). When **Shh** is mis-expressed in these regions using a pancreatic endothelium-specific promoter, the pancreatic mesoderm develops into smooth muscle typical of the intestine (ApeLvist et al., 1997). There is no effect, however, on the cell fates of the pancreatic endoderm in these mice, consistent with previous suggestions that **Shh** appears to be exclusively a signal from the endoderm to the mesoderm (Marigo et al., 1996).

An important set of possible target genes downstream of **Shh** in regionalizing the gut are the **Hox** genes. **Hox** genes are expressed in nested, overlapping patterns in the developing gut mesoderm. Moreover, the boundaries of expression of the 5’ members of the **Hoxa** and **Hoxd** clusters (paralogues 9-13) match the morphological borders of the different gut regions of the posterior midgut and hindgut (Roberts et al., 1995; Yokouchi et al., 1995). These expression patterns are initiated at the same time as **Shh** is expressed, as the gut tubes start to form at the CIP and AIP. **Shh** mis-expression can activate ectopic expression of these genes in the hindgut mesoderm (Roberts et al., 1995) suggesting that **Hox** gene expression is a response to this endodermal signal. The relevance of these **Hox** expression domains to differential gut morphogenesis has been supported by murine transgenic experiments in which inactivating mutations or misexpression results in abnormal gut development (for example, Pollock et al., 1992; Wolgemuth et al., 1989). In particular, loss of expression of **Hoxa-13** and **Hoxd-13**, which are normally expressed in the mesoderm of the rectum, result in the alteration of muscle layers of the sphincter consistent with a partial anterior transformation of this region (Kondo et al., 1996; Warot et al., 1997). In addition to their regionally restricted expression in the posterior-most gut mesoderm, these genes are also expressed throughout the hindgut endoderm. This is a feature unique to the 13th paralog as all the other **Hox** genes studied are restricted in their gut expression to the mesoderm layer alone without endodermal expression. Compound mutant mice carrying null alleles of...
both Hoxa-13 and Hoxd-13 show anomalies in the epithelial layers of the rectum in addition to the mesodermal malformations (Warot et al., 1997). Since these Hox genes are expressed in both tissue layers, it is impossible to determine whether the endodermal rectal phenotype is a direct effect of the loss of Hoxa,d-13 gene expression in the endoderm, or whether it is an indirect result of the lack of secondary mesodermally derived inductive signals, which are missing due to the absence of the Hox gene product in the mesoderm.

We have used viral misexpression in the chick embryo to further elucidate the roles of these factors in the epithelial-mesenchymal interactions which guide the regionalization of the gut. Although Shh conveys region-specific positional information, as in the specification of the pancreas, we find that the visceral mesoderm already possesses regional differences in its responsiveness to this signal, prior to the formation of the gut. Ectopic Shh induces Bmp4 throughout the mesoderm of the midgut, hindgut and the posteriormost foregut, but no induction of Bmp4 occurs in the gizzard or proventriculus. We find that ectopic expression of Bmp4 in the stomach regions causes a diminution of the normally thick mesodermal smooth muscle layers. Thus the regional restriction on Bmp4 induction by Shh in the vertebrate gut is likely important for the control of relative mesodermal growth in different regions. Similarly, the ability of Shh to induce Hoxd-13 expression is strictly limited to the regions of the developing gut posterior to the vitelline veins. Regionalized expression of Hox genes in the gut mesoderm, in turn, affects the region-specific differentiation of the adjacent endoderm in addition to the mesoderm itself; placing Hox genes upstream of the mesodermally derived signals involved in epithelial-mesenchymal interactions during gut development.

**MATERIALS AND METHODS**

**Embryos**

Chick embryos were obtained by incubation of fertilized White Leghorn eggs (SPAFAS) and were staged according to Hamburger and Hamilton (1951) and by day of incubation (embryonic day, E).

**Viral vectors**

Misexpression in the developing chick gut was achieved using a replication-competent retroviral vector – RCAS (Hughes et al., 1987). The viral constructs used have all been previously described, including vectors transducing the genes encoding Bmp4 (Duprez et al., 1996, a gift from Paul Brickell), Shh (Riddle et al., 1993), Hoxd-13 (Goff and Tabin, 1997) and (as a control) alkaline phosphatase (Fekete and Cepko, 1993). Viral production, harvesting, concentration and titering were carried out as described (Cepko, 1991).

**In ovo viral infection and culture**

Chick eggs between stages 7 and 13 (Hamburger and Hamilton, 1951), approximately 1.5-2 days of incubation, were windowed and viewed using an Olympus SZH dissection microscope, at room temperature. Using a micromanipulator, glass syringe and a pulled glass needle, approximately 0.1-0.5 µl of virus (titered at greater than 1x10⁸ CFU/ml) was injected into the specific regions of the presumptive gut mesoderm roughly following a published chick gut mesodermal fate map (Matsushita, 1995). Approximately 1-5 injections per embryonic side were performed. 750 ml of sterile PBS with penicillin and streptomycin was placed on the surface of the embryo prior to closing the egg window with tape. The eggs were allowed to sit undisturbed at room temperature following injection for at least 10 minutes before being placed into an egg incubator at 37°C with 50% humidified air.

Embryos were harvested at various time points (2-16 days postinjection) and analyzed for gross morphology. The embryos were subsequently fixed with 4% paraformaldehyde in PBS 12-18 hours. Fixed embryos were either processed for whole-mount in situ hybridization or for frozen or paraffin-embedded sections.

**Whole-mount in situ hybridization**

Embryos at stages earlier than E5 were processed intact. Older embryos were eviscerated manually and viscera were saved for analysis. The digoxigenin riboprobe synthesis and in situ hybridization techniques have been described previously (Riddle et al., 1993; Roberts et al., 1995).

**Sections for in situ hybridization, histology and immunohistochemistry**

Five to twenty µm sections were obtained from OCT compound (Miles, Inc., IN), gelatin or paraffin-embedded embryos or viscera and placed on superfrost plus slides (Fisher Scientific, PA). Sections were airdried from 1-18 hours and processed. Frozen sections were kept at -20°C until used. Hematoxylin and eosin (H and E) and Alcian blue pH 2.5 (AB) were prepared using standard procedures (Bancroft and Stevens, 1990). Frozen sections were used for in situ hybridization using digoxigenin-labeled riboprobes as previously described (Roberts et al., 1995). Immunohistochemistry was performed on OCT-embedded frozen sections following the protocol of Takahashi et al. (1986), using a chick monoclonal antibody to sucrase (a gift from T. Matsushita; Matsushita, 1984, 1996).

**Probes**

Many of the probes used for in situ hybridization in this study have been described in detail elsewhere, including Shh (Riddle et al., 1993), Ptc (Marigo et al., 1996), Hox genes (Burke et al., 1995), Nkx2.5 (a gift of T. Schultheiss; Schultheiss et al., 1995), CdxA (a gift of A. Fainsod; Frumkin et al., 1994), Wnt-5a (a gift of A. McMahon; Dealy et al., 1993), Bmp2 (Roberts et al., 1995) and Bmp4 (Roberts et al., 1995). Other Bmp probes were isolated using primers designed to amplify members of the TGF-β and Bmp families (Basler et al., 1993). Eight independent 120 bp BMP fragments were amplified from a stage 22 chicken posterior limb bud plasmid cDNA library. These fragments were pooled and used to screen an unamplified stage 22 limb bud Zap cDNA library constructed as in Riddle et al. (1993). Among the Bmp-related clones isolated were chicken Bmp5 (1600 bp), Bmp6 (2000 bp) and Bmp7 (3000 bp). All contain the full coding region. The probe for chick lumican was transcribed from a 161 bp fragment (nucleotide 246-405 of the cDNA).

**RESULTS**

**The visceral mesoderm displays regionalized, differential response to Shh**

Shh is expressed in the endoderm throughout the anteroposterior extent of the gut tube from the time it first forms at the AIP and CIP (Roberts et al., 1995; Fig 1B). The visceral mesoderm responds to Shh along the entire length of the gut tube, as evidenced by the expression of the target gene Ptc (Fig. 1D and Marigo et al., 1996). However, the consequences of Shh expression appear to differ between regions of the gut. For example, Shh induces intestine-specific mesodermal differentiation in the midgut (Apelqvist et al., 1997), yet other regions of the gut, such as the foregut, which do not form intestine, also express Shh. To test whether these
differences in response might reflect a pre-existing regionalization of competence within the splanchnic mesoderm, we examined the induction of two genes previously shown to be targets of Shh in the hindgut, Bmp4 and Hoxd-13 (Roberts et al., 1995), when Shh is misexpressed along the AP axis of the gut.

In wild-type chick embryos, Bmp4 is expressed throughout the mesoderm of the developing gut from the cloaca to the esophagus except in the stomach (gizzard and proventricularis) (Fig. 1C), suggesting that, like Ptc, its activation could be a universal response to Shh signaling in the gut mesoderm. In contrast mesodermal Hoxd-13 expression is limited to the posterior region of the hindgut at the forming cloaca (Fig. 1E,F).

Shh was misexpressed at various levels in the splanchnic mesoderm by infection with a retroviral vector prior to the closure of the gut tube. The infection protocol results in patches of infection, as seen by hybridization with a viral-specific riboprobe in Fig. 2A,B. As expected, ectopic Ptc is strongly induced throughout the gut mesoderm in regions that have been infected with Shh-expressing virus (Fig. 2C). The ability of the entire splanchnic mesoderm to respond to Shh is also apparent morphologically. Misexpression of Shh in the gut tube results in mass-like overgrowth in all regions that have been infected (Fig. 2C) distorting the shape of the gut organs by embryonic day 11 (Fig. 2E,F). Sectioning of infected guts demonstrates that the proliferative effect of Shh is limited to the mesodermal layer (Fig. 2D).

Since Bmp4 is expressed in mesoderm adjacent to Shh-expressing endoderm throughout the AP extent of the gut, we expected that it would be induced by Shh in all regions. As previously reported, misexpression of Shh induces Bmp4 in the hindgut and midgut (Roberts et al., 1995). Surprisingly, however, when Shh is misexpressed in the presumptive stomach, Bmp4 is not induced (Fig. 2G). Careful analysis of the rostral-caudal level at which we first observe Bmp4 induction reveals that this boundary coincides with the posterior-most region of the stomach. Shh misexpression that crosses this region results in a distinct line of Bmp4 induction (Fig. 2G). The related factors Bmp2, Bmp5 and Bmp6, were not found to be expressed in the stomach and were not induced when Shh was misexpressed (data not shown). Bmp7 is expressed in the dorsal stomach, but is not upregulated by ectopic Shh (data not shown). Thus, although Bmp4 is expressed throughout the gut tube, it can only be induced by Shh in the hindgut and midgut regions, not at the stomach region of the foregut.

A second border of Shh-responsiveness appears to lie at the midgut-hindgut boundary. Misexpression of Shh in presumptive hindgut endoderm and mesoderm results in induction of Hoxd-13 expression in the hindgut mesoderm, as previously reported (Roberts et al., 1995). However, we found that when Shh is misexpressed in the midgut or foregut, ectopic Hoxd-13 is not induced (Fig. 2H).

Ectopic Bmp-4 expression in the stomach limits mesodermal growth

We found the expression of Bmp-4 to be excluded from the gizzard and proventricularis. Moreover, ectopic Shh is apparently unable to induce Bmp4 in the mesoderm of this region. A possible clue to the reason for this regional restriction lies in consideration of the morphology of its mesodermal layer. The stomach region is distinct from the rest of the gut in having an extremely thick smooth muscle layer, especially in the gizzard. The rest of the gut has a relatively thin smooth
muscle component, 1/4-1/3 the thickness of the gizzard. We hypothesized that the role of Bmp-4 expression in the mesoderm of the developing gut may be to limit the mesodermal component outside of the stomach. To test this, we misexpressed Bmp-4 in the developing stomach region. This resulted in stomachs significantly smaller than wild type (Fig. 3B,C). Histological sections demonstrate that the small stomach phenotype is due to a loss of mass in the mesodermal component of the gizzard and proventricularis (Fig. 3D,E). This loss of mesoderm created such thin-walled stomachs that in many cases ulcerations of the stomach wall occurs.

**Mesodermal Hoxd-13 expression induces hindgut-specific differentiation of the adjacent endoderm**

In the context of the hindgut mesoderm, Shh induces the expression of Hoxd-13. To determine the role of Hoxd-13 in patterning the hindgut, we targeted misexpression of Hoxd-13 to the midgut mesoderm. Viral transgene expression (as detected by either RCAS-specific riboprobe or Hoxd-13 riboprobe) could be detected in the midgut mesoderm by E4 (Fig. 4A) and continued at least to E18 (Fig. 4B). Patchy expression was present in early stage embryos but, by E9-10, diffuse and confluent expression was seen. Usually the posterior 1/3 of the midgut and much of the hindgut mesoderm was infected. Importantly for interpreting the phenotype described below, misexpression was never detected in the midgut endoderm (Fig. 4C,D).

The morphologies of guts infected with Hoxd-13 and control alkaline phosphatase virus were compared to wild-type embryos. No gross alterations were observed in the position or relative lengths of the midgut, hindgut or cloaca. Histologic sections through the midgut of embryos infected with the Hoxd-13 virus revealed a consistent alteration in the endodermal phenotype. Normal midgut (small intestine) has an endodermal (mucosal) phenotype characterized by long thin villous projections into the lumen (Figs 4, 5A). These villi are present by E12 and are essentially mature by E18 (Romanoff, 1960). When Hoxd-13 is expressed in the midgut mesoderm at any level up to and including that of the pancreatic ductal epithelium, the endodermal morphology is altered. Villi are blunted, broad and flat, with occasional glands present (Fig. 5G). This mucosal phenotype resembles that normally found in the hindgut and cloaca (Fig. 5C-E). This phenotype was never observed following infection with the control virus (Fig. 5F,H). In contrast to the midgut, no mucosal alterations were observed in the hindgut of infected embryos (data not shown).

To verify that the phenotype that we observed was due to misexpression of Hoxd-13, affected embryos were assayed by in situ hybridization for viral-specific gene expression. The regions of midgut showing altered endodermal morphology were in all cases accompanied by viral expression in the mesoderm (for example, Fig. 4D). We also observed viral misexpression in the hindgut mesoderm of some embryos, in spite of the absence of a hindgut mucosal phenotype.

Expression of Hoxd-13 in the mesoderm thus affects the morphological differentiation of the adjacent endoderm. This could represent a specific regulation by Hoxd-13 of the production of an inductive factor involved in the mesenchymal-epithelial interaction. Alternatively, it could reflect general conversion of the midgut mesoderm to hindgut mesoderm. To examine this possibility, we looked at the expression of a series of transcription factors normally expressed in a region-specific

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**Fig. 2.** (A) Schematic of the embryonic foregut and midgut at E4 showing rostral-caudal regionalization. Schematic represents orientation of guts used in this figure. Abbreviations are the same as in Fig. 1A. (B) After viral misexpression of Shh in the foregut/midgut regions of a stage 10 chick embryo, harvest at E4 and demonstration of viral expression by whole-mount in situ hybridization. Signal is present in the posterior foregut (stomach) and midgut. (C) Embryo treated as in B but hybridized with a riboprobe to Ptc showing increased expression in regions of the stomach. (D) Section through an E10 embryo (as in C) showing the overgrowth of tissues is restricted to the mesoderm (smooth muscle) – black arrowhead. Purple arrowhead at endoderm of gizzard. (E,F) Eviscerated stomachs of E10 embryos. (E) Wild type with proventricularis and gizzard. (F) Embryo with ectopic expression of Shh as in A showing massive overgrowth of tissues distorting normal morphology. (G) Eviscerated stomach and intestines from an embryo treated as in B, harvested at E10, and hybridized with riboprobes for Shh (magenta) and Bmp4 (purple/black). Ectopic Shh does not induce Bmp4 within the stomach (anterior to white arrowhead), but ectopic Shh does induce Bmp4 in the midgut (purple arrowhead). Red arrowhead indicates positive hybridization for Bmp4 in lungs. (H) Embryo treated as in B harvested 6 hours after injection and hybridized with riboprobes for Shh (dark magenta) and Hoxd-13 (blue/black). Ectopic Shh does not induce Hoxd-13 in the anterior midgut (red arrowhead) or foregut but does in the posterior midgut/hindgut (black arrowhead).
manner in the mesoderm. The Hox genes from the 5' ends of the A and D clusters are expressed in a nested set in the hindgut mesoderm of the developing gut (Roberts et al., 1995). We examined the expression of Hoxd-10, Hoxd-12, Hoxa-10, Hoxa-11 and Hoxa-13, but none were induced in the midgut of Hoxd-13-infected embryos (data not shown). Conversely, Hoxa-9 and Hoxd-9, which are normally expressed in the mesoderm of the developing midgut, are not down-regulated in response to Hoxd-13 misexpression (data not shown). Thus, there does not appear to be a general conversion of the mesoderm to a hindgut phenotype in response to Hoxd-13.

Following Hoxd-13 misexpression, the midgut mesoderm retains its normal region-specific expression pattern of other Hox genes. Therefore, one would predict that the infected midgut might continue to produce its normal endogenous signals to the adjacent endoderm in addition to whatever signals are ectopically made in response to Hoxd-13 misexpression (data not shown). Thus, there does not appear to be a general conversion of the mesoderm to a hindgut phenotype in response to Hoxd-13.

Factors expressed differentially at the hindgut-midgut boundary include two secreted proteins. Wnt5a has been previously reported as being expressed in the embryonic gut (Dealy et al., 1993). We found Wnt5a expression restricted to the mesoderm of the developing midgut (data not shown). Conversely, Hoxa-9 and Hoxd-9, which are normally expressed in the mesoderm of the developing midgut, are not down-regulated in response to Hoxd-13 misexpression (data not shown). Thus, there does not appear to be a general conversion of the mesoderm to a hindgut phenotype in response to Hoxd-13.

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Since the midgut mesoderm continues to produce at least some of its normal signals in the presence of Hoxd-13, it would be expected that the responding midgut endoderm is exposed to both midgut- and foregut-specific inductive factors and, hence, be mixed in character, in spite of its overt hindgut-like morphology in infected regions. This indeed appears to be the case. Both Nkx2.5, which is normally expressed in the anterior midgut endoderm (Buchberger et al., 1996; Lints et al., 1993),
and CdxA, which is normally strongly expressed in the midgut endoderm with a lower level of expression in the hindgut mesoderm (Frumkin et al., 1993, 1994), are unchanged in their expression patterns following Hoxd-13 misexpression (data not shown). Similarly, the digestive enzyme sucrase, which is normally produced in the midgut but not the posterior hindgut, is present in its normal distribution (Fig. 6). In contrast to the retention of these normal distributions of midgut-specific gene products, we found that hindgut-specific endodermal markers were ectopically produced in the midgut endoderm following Hoxd-13 infection of the midgut mesoderm. The hindgut and cloacal epithelial cells contain predominantly acid mucins but the midgut epithelium contains predominantly neutral mucins. By staining for acid mucins, the differences between two regions is easily discerned (Fig. 7). Following Hoxd-13 misexpression, the hindgut production of acid mucins, seen in frozen sections of E18 embryos, was unaltered. However, in Hoxd-13-expressing midguts, epithelial cells have an acid mucin pattern resembling the hindgut or cloaca (Fig. 7A). The average number of acid-mucin-producing cells in the midgut is increased from a wild type of 13-30 per mm to 70-77 per mm. Hindgut and cloacal acid-mucin-producing cells number 64-127 per mm. Thus, in the presence of Hoxd-13 in the mesoderm, inductive interactions lead to the endoderm adopting a gross hindgut morphology and expression of at least some aspects of hindgut cytodifferentiation.

DISCUSSION

The formation of any complex organ requires a high degree of coordination between the different tissue layers from which it forms. Patterning the gut has been shown to involve reciprocal inductive signaling between the endoderm and mesoderm during development (Kedinger et al., 1986). We have investigated aspects of that signaling during the process of AP regionalization of the gut tube. Shh is a secreted factor made by the endoderm as the gut tube first forms. Exposure to Shh results in the expression of genes in the mesoderm, such as the Hox genes, which likely act to demarcate functional domains of the gut (Roberts et al., 1995). However, the response to Shh is itself regionally restricted. Apparently Hoxd13 is inducible only in the gut mesoderm, which originated from the CIP (that posterior to the vitelline veins). Bmp4 cannot be induced in the foregut region, which will form stomach. As Shh was ectopically expressed before the gut tube had formed, these
conjunction with additional inductive factors, which are either there is an AP prepattern in the splanchnic mesoderm or Shh influence of Regionalized response to Shh cytodifferentiation and morphogenesis.

Once Hoxd-13 is expressed in the visceral mesoderm, it appears to regulate subsequent signaling back to the endoderm, which leads to the induction of hindgut-specific cytodifferentiation and morphogenesis.

**Regionalized response to Shh**

The differential response of target genes to the inductive influence of Shh in the foregut, midgut and hindgut implies that either there is an AP prepattern in the splanchnic mesoderm or Shh acts on the mesoderm to induce these targets only in conjunction with additional inductive factors, which are themselves spatially restricted. Consistent with the first hypothesis, several genes have been reported to have distinct boundaries of expression in this region of the gut, for example: Barx1 in mouse, and Raldh-2 and Nkx2.3 in chick. Barx1 is a homeodomain-containing transcription factor found in the mesoderm of the stomach (Tissier-Seta et al., 1995). Raldh-2 is an enzyme involved in retinoic acid synthesis and is found in the mesoderm of the small and large intestine, but not in the stomach mesoderm (Niederreither et al., 1997). Nkx2.3 is a homeodomain containing transcription factor limited to the mesoderm of the small and large intestines (Buchberger et al., 1996). The timing of expression of these genes relative to that of Shh has not been explored.

**Bmp4**, which can be ectopically induced by Shh throughout the hindgut/midgut region, is indeed normally expressed in mesoderm adjacent to Shh-producing endoderm throughout this region. In contrast, we find that, while mesodermal Hoxd-13 can be induced by Shh throughout the hindgut, it is normally expressed only in the caudal-most hindgut, the cloaca. This regional restriction could be explained by the presence of a specific time window during which cells are competent to initiate Hoxd-13 expression. Shh is first produced at the CIP, where the posterior gut tube originates. This is, indeed, where Hoxd-13 is induced and the future cloaca develops. As the endodermal invagination spreads anteriorly and the tube elongates, Shh expression is coordinately activated in the endoderm. However, this may be past the time when the more anterior hindgut is capable of responding by activating Hoxd-13. The Abd-B-like Hox genes of the A and D clusters are all regionally restricted in their expression very early in gut development, before the CIP has formed a tube but after Shh is expressed at the CIP region (Roberts et al., 1995; Yokouchi et al., 1995), and their expression may be similarly regulated by a combination of the inductive influence of Shh and timing of cell competency.

**Bmp4** expression is activated in the adjacent mesoderm throughout the hindgut and midgut. However, Bmp4 is not endogenously expressed in the mesoderm of the stomach and is not induced when Shh is misexpressed in this region. A distinct boundary line is seen wherein Bmp4 cannot be upregulated on one side of the boundary (stomach), while upregulation can occur on the other side of the boundary (duodenum).

One functional consequence of the differential induction of Bmp4 may be to regulate the thickness of the mesodermal layer in different regions of the gut. We find that a result of ectopic Shh expression is a mass-like tissue overproliferation. This action of Shh is limited to the mesoderm as ectopic Shh had no effect upon the endoderm. This is not surprising in that Ptc, part of the receptor complex for Shh, is not expressed in the endoderm (Marigo et al., 1996). Bmp4, which is expressed throughout most of the gut mesoderm, could serve to negatively modulate the proliferative increases caused by Shh, thus, preventing overproliferation of the mesoderm beyond that which is normal for that gut region. Therefore, in regions in which Bmp4 is not present, one would expect to have much thicker mesoderm than in regions that do have Bmp4 present. This is consistent with the wild-type morphology of the chick gut. The gizzard, which does not express Bmp4, has very thick mesoderm compared to the lower intestinal regions. The gizzard has three layers of musculature while the intestine has but two. That this thick musculature is established in part by
restricting the domain of Bmp4 expression is supported by the finding that, when Bmp-4 is ectopically expressed in the developing stomach region, the mesodermal component is significantly diminished giving a small stomach phenotype.

**Hoxd-13 regulates signaling from the mesendoderm to the endoderm**

We focused here on the functional consequences of the activation of a second Shh target, Hoxd-13. In the normal chick gut, Hoxd-13 mesendodermal expression is restricted to the most posterior aspect of the gut (Roberts et al., 1995; Yokouchi et al., 1995). In the chick embryo, this region is the cloaca (the common gut/urogenital cavity). Although the mature cloaca has three compartments (from proximal to distal): coprædeum, urodeum and proctodeum; until hatching the cloaca is subdivided into two functional groups based on their ability to recognize specific DNA targets in a complex with Pbx1a (Shen et al., 1997). Hox paralogues 11, 12 and 13 form a distinct functional group, and these are precisely the genes that are expressed exclusively in the hindgut.

Misexpression of Hoxd-13 results in a histological transformation and an alteration of mucin expression in the midgut. However, following misexpression, the midgut epithelium retains other characteristics typical of that region, including expression of region-specific transcription factors and production of the digestive enzyme sucrase. Some of these midgut-specific patterns may be regulated independently of mesodermal inductive signals. Indeed, previous co-culture experiments have indicated that some aspects of regionalized epithelial differentiation are determined by the adjacent mesoderm while others are not. For example, the upper stomach (proventricularis) secretes a specific enzyme: pepsinogen (Hayashi et al., 1988; Takiguchi et al., 1986, 1988). Small intestinal epithelium has a microscopic morphology distinct from the proventricularis and expresses enzymes distinct from the proventricularis, such as sucrase, and does not express pepsinogen. When presumptive small intestinal epithelium is cultured with proventricular mesoderm, the epithelium develops a morphology that is glandular (proventricular) but the cells do not express pepsinogen and they retain their expression of sucrase (Hayashi et al., 1988; Yasugi, 1984; Yasugi et al., 1985, 1991). While some of the midgut epithelial characteristics may thus be patterned cell autonomously, other aspects of the mixed endodermal phenotype seen following Hoxd-13 misexpression are very likely to be due to the continued expression of midgut-specific inductive factors by the infected mesoderm in addition to those induced by Hoxd-13. We did not observe any downregulation of the midgut mesodermal markers that we examined.

This study has elucidated several aspects of mesenchymal-epithelial interaction during gut regionalization and differentiation. Our findings provide evidence that differential responsiveness as well as differential signaling plays a role in patterning the gut; and that Hox genes, initially induced in response to such signaling, subsequently mediate the production of region-specific cues, forming part of the dynamic instructive cross-talk between tissue layers in the forming gut.

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Pattern formation in the developing chick gut


