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Comparison of Acid Mucin Goblet Cell Distribution and *Hox13* Expression Patterns in the Developing Vertebrate Digestive Tract

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ABSTRACT The digestive tract of vertebrates is a complex organ system required for the digestion of food and the absorption of nutrients. The colon evolved as a water absorption organ essential for vertebrates to survive on land. In contrast to land vertebrates, the Chondrichthyes (sharks, skates and rays) are nearly iso-osmotic with their ocean environment and do not reabsorb water from food waste. To understand the origin of the vertebrate colon, we examined the distribution of sulfated and sialyated mucus-producing cells in the little skate, *Raja erinacea,* as an indication of water absorption function in the chondrichthian digestive tract. The percentage of acid mucin producing goblet cells was analyzed in the spiral valve and hindgut of little skate and the small intestine and colon of mouse embryos. Levels of acid mucins in the hindgut of the little skate was comparable to that of the small intestines of terrestrial vertebrates, whereas the distal region of the spiral valve contained high levels of acid mucin producing cells similar to the colon of mouse and chick. The low numbers of acid mucins in the little skate hindgut confirms that a functional colon for water absorption is absent in the Chondrichthyes. Interestingly, the presence of high levels of acid mucins in the posterior spiral valve provides evidence for a possible primordial water-absorbing organ in the elasmobranchs. *Hoxd13* patterns acid mucins in the colons of terrestrial vertebrates. Expression of *Hoxd13* and *Hoxa13* in *R. erinacea* suggests conserved roles for *Hox* genes in patterning the early hindgut. *J. Exp. Zool. (Mol. Dev. Evol.) 308B:442–453, 2007.*

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The Chondrichthyes including sharks, skates and rays are cartilaginous marine fish that have served as biomedically relevant models of physiology as well as of comparative genomics in recent years (Ballatori et al., 2003; Mattingly et al., 2004; Barnes et al., 2005). The cartilaginous fish appeared approximately 450 million years ago and thus constitute a primitive vertebrate useful for physiological studies. Studies in the spiny dogfish (*Squalus acanthias*) and little skate (*Raja erinacea*) include function of the cystic fibrosis transmembrane conductance regulator (CFTR), the Na/K-ATPase, and the liver (Ballatori and Villalobos, 2002; Ballatori et al., 2003; Bewley et al., 2006; Cai et al., 2003; Ratner et al., 2006; Weber et al., 2006). In these fish, the digestive tract is of particular interest due to its ability to adapt to a predatory diet and simultaneously maintain methods of buoyancy and osmoregulation.

The major subclass of cartilaginous fish, Elasmobranchii, feed primarily on teleosts. It is important that the chondrichthian digestive tract is able to extract the required nutrients from its food efficiently because space is limited within the animals' body cavity for placement of visceral organs. Narrow body cavities, combined with large livers utilized for buoyancy, and the retention of embryos within the body, (in the case of oviparous females), all contribute to the need for compact and efficient digestive structures in the chondri-

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chythians. Sufficient digestion is ensured by the increased digestive surface area provided by the spiral valve intestine in these species (Wetherbee et al., '90). The spiral valve forms from folds within the submucosa of the intestine, creating the appearance of a corkscrew (Fig. 1B and C) (Wetherbee et al., '90). Villi cover both surfaces of the folds, allowing increased nutrient absorption in a short stretch of intestine that connects the stomach with the hindgut (Figs. 1D and 3A). The spiral valve, which is not found in teleosts, is an example of how unique structures evolved as a result of diet and anatomic limitations in the digestive tract (Fig. 1A).

In addition to the unique spiral valve structure, the digestive tracts of Chondrichthyes are functionally unique due to their method of osmoregulation. The cartilaginous fish maintain a body osmolarity that is slightly higher than the surrounding ocean environment (Randall et al., '97). They accomplish this through a renal tubule system that retains high levels of urea and trimethylamine oxide (TMAO); TMAO neutralizes the denaturing effects on proteins of high urea levels (Holmes and Donaldson, '69; Randall et al., '97). The slight relative elevation in osmolarity of the Chondrichthyes allows them to obtain water by passive diffusion through the gills (Janech et al., 2003). In terrestrial vertebrates, the large intestine and colon (defined as encompassing the hindgut) function primarily to concentrate an organism's stool against a strong osmotic gradient. However, in the Chondrichthyes reclaiming water from the feces may play a less significant role because of passive diffusion through the gills. This brings into question the function of the hindgut in cartilaginous fish, as these regions do not appear to function similarly to other vertebrates. Different

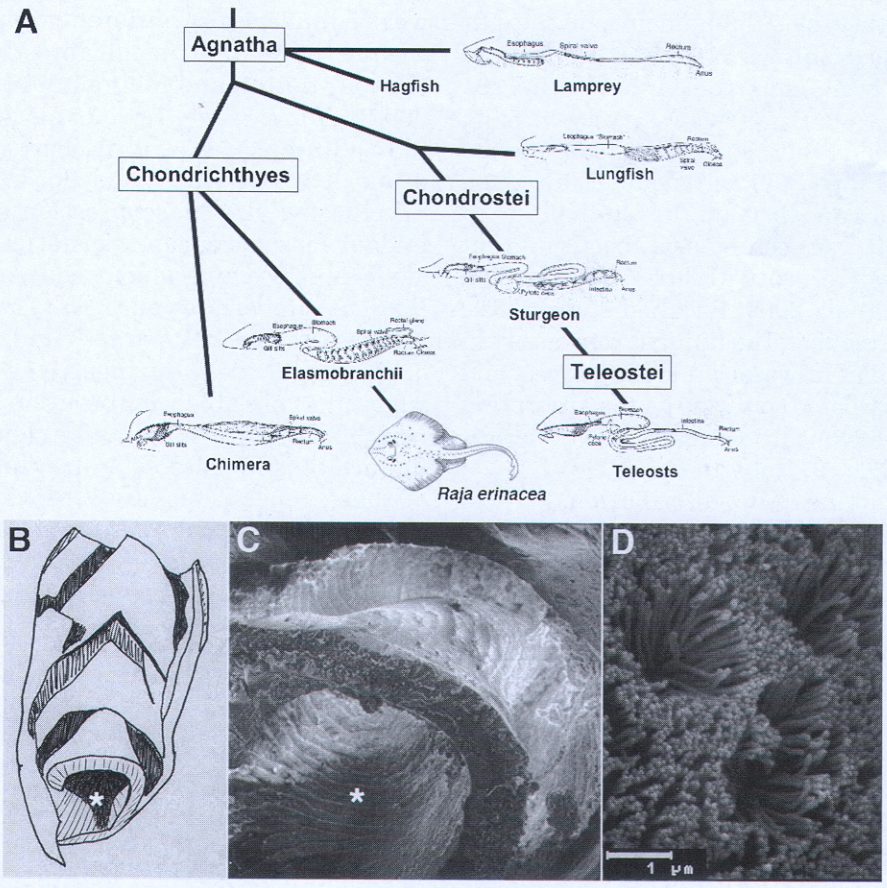


Fig. 1. Phylogeny of craniates with spiral valve intestines. (**A**) Spiral valve intestines are found in several classes of craniates including Agnatha (lamprey), Chondrichthyes (chimera, sharks, skates and rays) and Chondrostei (sturgeon). Spiral valve intestines are absent from Teleosts. [Adapted from Romer and Parsons ('85)] (**B**) Cartoon drawing of a freeze-fractured spiral valve from a *Squalus acanthias* (dogfish) embryo, revealing the inner layers of the corkscrew-like structure. Asterisk indicates the center lumen of the spiral valve. (**C, D**) Scanning electron micrographs of the spiral valve cartooned in (B). (C) View looking into the spiral valve (central lumen, asterisk). (D) Cilia are visible on the surface of the coils of the spiral valve in (C).

digestive systems specialized to unique environments can be characterized by specific subpopulations of cell types. Mucosubstrates within the epithelial lining of the gut tube has been used to assess and characterize organ function across different species (Filipe, '79; Slomiany et al., '80; Aksoy and Akinci, 2003).

Mucosubstrates, or mucins, serve to protect the epithelial lining of the digestive tract from mechanical and chemical damage, while supporting the transfer of nutrients and water into absorptive cells in the epithelium (Smith, '36; Filipe, '79; Lichtenberger, '95; Lugea et al., 2000). The composition of carbohydrate side chains of mucins varies within the digestive tract, according to the function of each organ (Sinha and Chakravorty, '82). Sulfate and sialic acid side chains confer anionic character to mucins and are found in increasing concentrations toward the distal region or colon of the human digestive tract (Filipe, '79; Buisine et al., '98; Corfield et al., 2000). The presence of sulphomucins in the gut epithelium supports the various types of bacteria and other microorganisms that inhabit the gut (entering by ingestion), as well as the varying absorptive functions of specific regions of the digestive tract (Filipe, '79; Corfield et al., '92). Levels of mucins can vary in the epithelium in accord with diet and commensal bacteria that inhabit the digestive tract (Filipe, '79; Corfield et al., '92; Lugea et al., 2000; Backhed et al., 2005). Therefore, acid mucins in the hindgut may assist in the efficient transfer of water and electrolytes, and thus has been correlated with the water absorption function of the colon in terrestrial vertebrates (Reifel and Travill, '79; Roberts et al., '98).

This study examines the distribution of acid mucins in the Elasmobranchii digestive tract to evaluate the function of the different regions of the spiral valve and colon (Roussel and Delmotte, 2004). The quantified distribution of acid mucin-producing goblet cells was analyzed during two stages of *R. erinacea* life history. Embryonic stages were examined to assess the distribution of mucosubstrates as patterned by early developmental signals, and compared to distributions in the mouse embryo. Since the introduction of bacteria into the digestive tract can influence the presence of mucosubstrates in the epithelium, we also examined the levels of acid mucins in the spiral valve and hindgut of *R. erinacea* post-embryonic day 45 animals. By analyzing the distribution of acid mucins in the little skate digestive tract, we are able to predict the possible functions of the regions along the gut tube, and speculate on the origin of the water-absorptive colon found in higher terrestrial vertebrates.

The *Hox* gene cluster regulates patterning of the embryonic body plan including the developing gut tube (McGinnis and Krumlauf, '92; Grapin-Botton and Melton, 2000; Kawazoe et al., 2002). During development, the early gut tube becomes regionalized along its anterior–posterior axis to ultimately give rise to the functionally distinct organs along the digestive tract. The gut tube is composed of two layers: an inner endoderm-derived epithelium surrounded by an outer splanchnic mesoderm. The expression of *Hox* genes with the genomic cluster displays colinearity with expression along the anterior to posterior body axis corresponding to a 3′ to 5′ position along the cluster. In the gut tube, the *Hox* genes are expressed in overlapping regions in the developing gut mesoderm. The boundaries of expression for the 5′ *Hoxa* and *Hoxd* clusters (comprising paralogs 9–13) correspond with boundaries of different organ morphologies within the midgut (small intestine) and hindgut (Grapin-Botton and Melton, 2000; Kawazoe et al., 2002). *Hoxa13* and *Hoxd13* pattern the hindgut (de-Santa-Barbara and Roberts, 2002; Roberts et al., '98) (Kondo et al., '96; Warot et al., '97). Specifically, viral misexpression of *Hoxd13* in the midgut mesoderm can transform the endoderm to a morphology and mucin content similar to the hindgut (Roberts et al., '98). Thus, we examined the expression of *Hoxa13* and *Hoxd13* in the developing *R. erinacea* digestive tract. Our results suggest a possible conservation of *Hoxa13* and *Hoxd13* for patterning the hindgut, although with implications for altered transcriptional regulation of these genes. This study provides the basis for future studies on the elasmobranch digestive tract, and understanding the regulation of morphologic changes during evolution of digestive tract development.

MATERIALS AND METHODS

Embryos

Raja erinacea (little skate) egg cases were obtained from Woods Hole Marine Biological Laboratory (Woods Hole, MA) and cultivated in circulating salt-water marine tanks at 13°C with a salinity of 32 ppt. Embryos were staged based on the presence of internalized gill structures, size of remaining yolk sac, and development of fin buds. Although there is no literature available for embryonic staging of *R. erinacea*, early morpholo-

gical development of little skate correlated with staging schemes from different chondrichthian lineages (Didier et al., '98). In this study, little skate embryo staging was approximated using stages from *Scyliorhinus canicula* (lesser spotted dogfish) (Ballard et al., '93). Fertilized White Leghorn chick eggs were obtained from SPAFAS (North Franklin, CT) and staged according to Hamburger and Hamilton (Hamburger and Hamilton, '51).

SEM

For scanning electron microscopy, spiny dogfish (*Squalus acanthias*) embryos were obtained from Mount Desert Island Biological Laboratory (Salisbury Cove, ME). The digestive tracts were removed and regions of the spiral valve were freeze-fractured under liquid N_2 in both longitudinal and transverse orientations. Spiral valve pieces were then dehydrated to 100% ethanol, critical point dried, and coated in gold in preparation for SEM analysis with a Zeiss DSM 950 microscope.

Histochemistry

Digestive tracts from *R. erinacea* stage 33 (just before hatching), post-embryonic day 45 (P45) and mouse embryonic day (*E*) 17.5 embryos (SW, Charles River Laboratories, Inc., Cambridge, MA) were fixed in 4% paraformaldehyde and dehydrated to 100% ethanol (Ballard et al., '93). Digestive tracts were embedded in paraffin and cut into 6 μm sections. Acidic mucosubstrates were stained for 10 min with alcian blue (pH 2.5) and counterstained with nuclear fast red (Sheehan and Hrapchak, '80). Alcian blue at pH 2.5 stains weakly sulphated mucosubstrates as well as carboxyl groups of sialomucins (Filipe, '79). Epithelial cells in four different 600 × fields were counted throughout the length of the spiral valve and hindgut in little skate, and small intestine and colon in mouse. Microsoft Excel was used to produce histograms comparing percentages of acid mucins between different regions of the embryonic and post-embryonic little skate digestive tract.

Isolation of R. erinacea Hoxa13 and Hoxd13

We isolated a fragment of *Hoxa13* (320 base pairs) and full-length transcript of *Hoxd13* (1,225 base pairs) from complementary DNA prepared from a stage 30 embryo using degenerate primers. The amino acid sequences of the degenerate primers used to amplify *Hoxa13* were FGSGYY and QMYCPK (GenBank accession number EF060198). The *Hoxd13* transcript was amplified in two fragments with degenerate primers MDGLGGN and LLSMEGY, and LLSMEGY and VKDNIP, isolating the 5′ and 3′ends, respectively. The fragments were assembled to create a full-length *Hoxd13* by taking advantage of a unique *Nco1* site in the LLSSMEGY sequence (GenBank accession number EF060197).

Whole mount RNA in situ hybridization

R. erinacea (stage 27) and chick (HH stage 26) embryos were removed from their egg cases and dissected away from the yolk sacs (Hamburger and Hamilton, '51; Ballard et al., '93). To stain the digestive tracts completely, the ventral body wall and liver were removed. Embryos were fixed in 4% paraformaldehyde/PBS pH 7.4 for 12 hr at 4°C, dehydrated to 100% methanol and stored at −20°C until used. Whole mount in situ hybridization was performed using digoxigenin-labeled riboprobes (Riddle et al., '93; Burke et al., '95), with the following modifications for little skate embryos: hybridization wash at 70°C in 50% formamide; 1.3 × SSC pH 4.5; 5 mM EDTA pH 8; 50 ug/ml tRNA; 0.2% Tween-20; 0.5% CHAPS; 100 ug/ml heparine; and post hybridization washes at 70°C in Solution 1 (50% formamide; 1.3 × SSC; 0.2% Tween-20) and Solution 2 (50% formamide; 1.0 × SSC; 0.2% Tween-20) (Sauka-Spengler et al., 2001). Probes used for in situ hybridization included *cHoxa13* (Burke et al., '95), *cHoxd13* (Burke et al., '95), *ReHoxa13* (MGID Number 3837), and *ReHoxd13* (EF060197). For discerning domains of expression, whole mounted embryos were embedded in 7.5% gelatin in 15% sucrose /PBS (Stern and Holland, '93). Gelatin blocks were flash frozen in 2-methylbutane on dry ice and 20 μm sections were cut and mounted in Gelvatol (Rodriguez and Deinhardt, '60).

RESULTS

The spiral valve intestine provides a unique organ for studying morphologic changes during evolution of digestive tract development. There are three classes of craniates that possess spiral valves (Fig. 1A). Of the most evolutionarily distant craniates (Agnatha), the lampreys but not the hagfish have valvular intestines that consist of loose coils of epithelium (Stevens and Hume, '95). The elasmobranchs, which include sharks, skates and rays have ordered spiral valves that vary in

the number of regions separated by an internal coil structure (Fig. 1B and C). The *R. erinacea* spiral valve when sectioned in transverse, contains eight regions or coils that decrease in volume distally, with the most posterior compartment containing the least volume (Fig. 3A). The *R. erinacea* spiral valve, like *Squalus acanthias*, is lined with villi on all surfaces of coils 1–6 (Fig. 1D and 3A). In the final posterior coils (numbers 7 and 8) the villi pattern is replaced with a more glandular simple columnar epithelium reminiscent of the ileo-caecal junction in higher vertebrates (Fig. 3A and C) (Burkitt et al., '95). The little skate hindgut contains squamous epithelium characteristic of the human anus and chick cloaca (Fig. 3D) (Burkitt et al., '95; Roberts et al., '98).

Acid mucins in embryonic and P45 R. erinacea midgut and hindgut

The distribution of acid mucins throughout the gut of an organism is an indication of the function of the different regions (Roussel and Delmotte, 2004). The presence of acid mucins in the hindgut has been correlated with the water absorption function of the colon in terrestrial vertebrates (Filipe, '79; Roberts et al., '98). Based on the unique osmotic and ionic regulatory characteristics of the Chondrichthyes, it is possible that the colon or hindgut does not function to absorb water as in teleosts and terrestrial vertebrates. If the hindgut region in Chondrichthyes does not function to extract water from the feces, then it should exhibit a corresponding absence or decrease in acid mucins. This hypothesis was tested by examining the distribution of acid mucins in the *R. erinacea* digestive tract.

The number of sialo and sulfomucins were counted and compared to the total number of epithelial cells along the length of the spiral valve intestine and hindgut of stage 33 *R. erinacea* embryos (Ballard et al., '93). The percentages of acid mucins were calculated in 600× fields randomly selected from each coil of the spiral valve intestine. Using the percentage of acid mucins allowed the analysis of general acid mucin distribution trends, as opposed to comparing absolute numbers, which may be skewed by the decrease in volume of the coils. The ratio of acid mucin-producing cells to total cells was tabulated for each coil of the spiral valve as indicated in (Fig. 3A). In the anterior half of the spiral valve, very few acid mucin-producing cells were detected (0.8–2.3% in coils 1–5) (Figs. 2 and 3A). A gradual increase in mucins was detected from coil 6, increasing to 18.8% of epithelial cells in the posterior-most coil (coil number 8) of the spiral valve (Figs. 2 and 3C). In the embryonic little skate hindgut, only 3.3% of epithelial cells contained acid mucin secreting vacuoles (Figs. 2 and 3D).

Since the levels of mucins can vary in the epithelium with diet and the presence of commen-

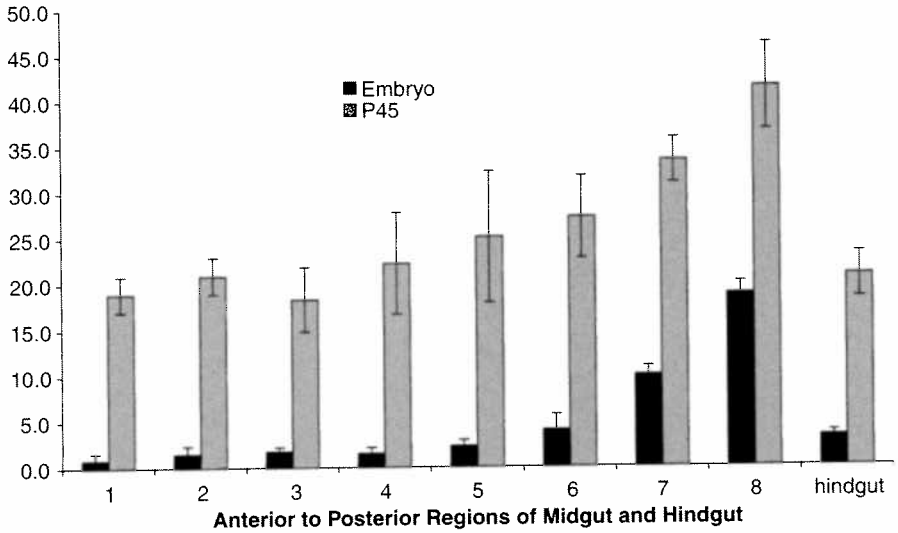


Fig. 2. Comparison of acid mucin distribution in the spiral valve and colon of embryonic and post-hatching stages of *R. erinacea*. Represented are the average percentiles of epithelial cells containing acid mucin producing-goblet cells from the different coils of the spiral valve and colon. Regions 1–8 on the *x*-axis correspond to the coils numbered from anterior to posterior in Fig. 3A. Standard deviation bars are indicated.

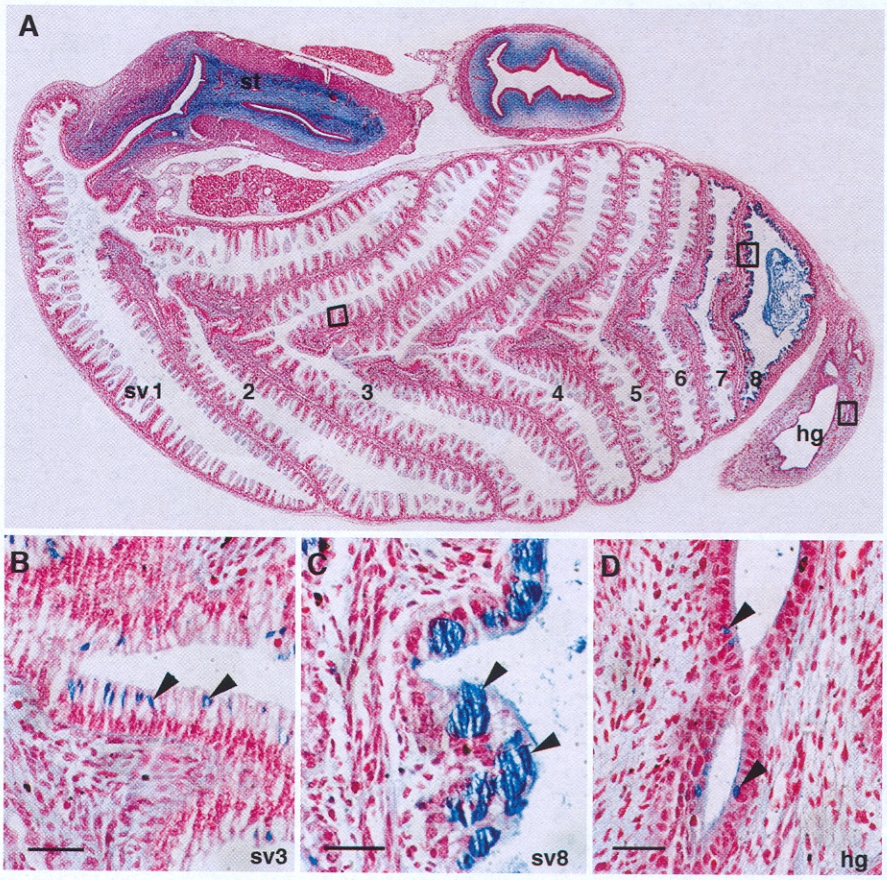


Fig. 3. Presence of acid mucin-producing goblet cells in the digestive tract of *R. erinacea*. Six micron sections through the digestive tract of a pre-hatched *R. erinacea* embryo (**A–D**) stained with alcian blue and nuclear fast red. (A) Photomerge of sections through the stomach, spiral valve and hindgut of a digestive tract at stage 33 (approximately 9 months of development). Eight continuous coils through the spiral valve are numbered. Boxes in coils 3, 8, and hindgut correspond to magnified sections in (B–D), respectively. (B–D) Alcian blue stained acid mucin-containing vacuoles (arrowheads) are clearly visible in contrast to nuclear red stained nuclei. st, stomach; sv, spiral valve; hg, hindgut.

sal bacteria that inhabit the digestive tract, we examined the acid mucin content of hatched animals (Filipe, '79; Backhed et al., 2005). Our assumption was that by post-embryonic day 45 the digestive tracts would be colonized by ingested bacteria and microorganisms. Compared to the embryo, the hatched little skate had elevated numbers of acid mucins for all regions (Fig. 2). In coils 1–5, the acid mucin content was approximately 20% in the hatched animal, compared with 2% or less in the embryo. Although the overall numbers of acid mucins were higher in hatched *R. erinacea* when compared to the embryo, a similar distribution was observed in both, with the highest concentration of acid mucins (41.5% and 18.8%, respectively) found in the final coil (number 8) of the spiral valve (Fig. 2). The numbers of acid mucins in the colon likewise dropped to levels seen in the proximal spiral valve (20.9% and 3.3%, respectively). Although the distribution profiles for acid mucins appear similar in embryonic and hatched animals, the increase in acid mucins seen in the hatchling may be due either to maturation of the digestive tract or exposure to a larger variety of colonizing bacteria resulting from the ingestion of marine water in the hatched animals.

Acid mucin distribution in E17.5 mouse small and large intestines

For comparison to a land vertebrate, we determined the distribution of acid mucins in the small and large intestines of an E17.5 mouse embryo. In the small intestine, approximately 1.5% of epithelial cells contained acid mucin secreting vacuoles (Fig. 4A), while 24% of cells were found in the large intestine (Fig. 4B). This distribution was consistent with what has been demonstrated for

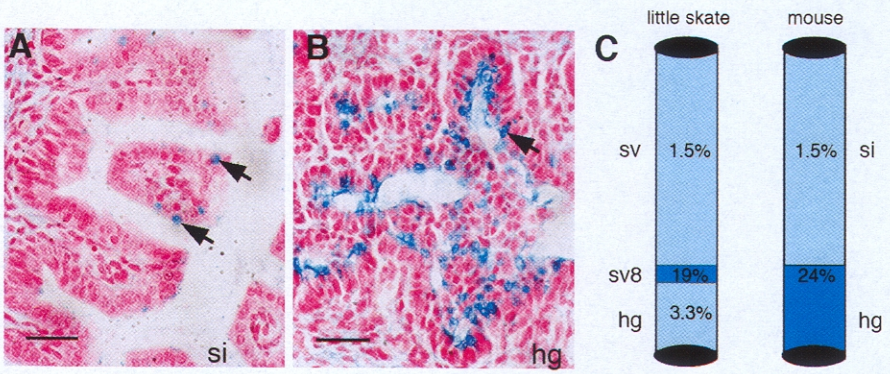


Fig. 4. Acid mucin-producing goblet cells in the embryonic mouse digestive tract. Six micron sections through the digestive tract of an E17.5 mouse embryo (**A**, **B**) stained with alcian blue and nuclear fast red. (A) Few alcian blue positive cells (1.5%) are found in the small intestine epithelium. (B) High density of acid mucin producing cells (24%) is found in the hindgut epithelium, corresponding to the water absorbing function of the colon. (**C**) Schematic comparing the distribution of acid mucin goblet cells in the little skate and mouse embryos. si, small intestine; sv, spiral valve intestine; hg, hindgut.

chick (33% of cells in the large intestine) (Roberts et al., '98). The distribution of acid mucins in the terrestrial vertebrate colon contrasts significantly with the distribution found in *R. erinacea*, suggesting differing roles for the chondrichthian and terrestrial vertebrate hindguts (Fig. 4C) (Filipe, '79; Roberts et al., '98).

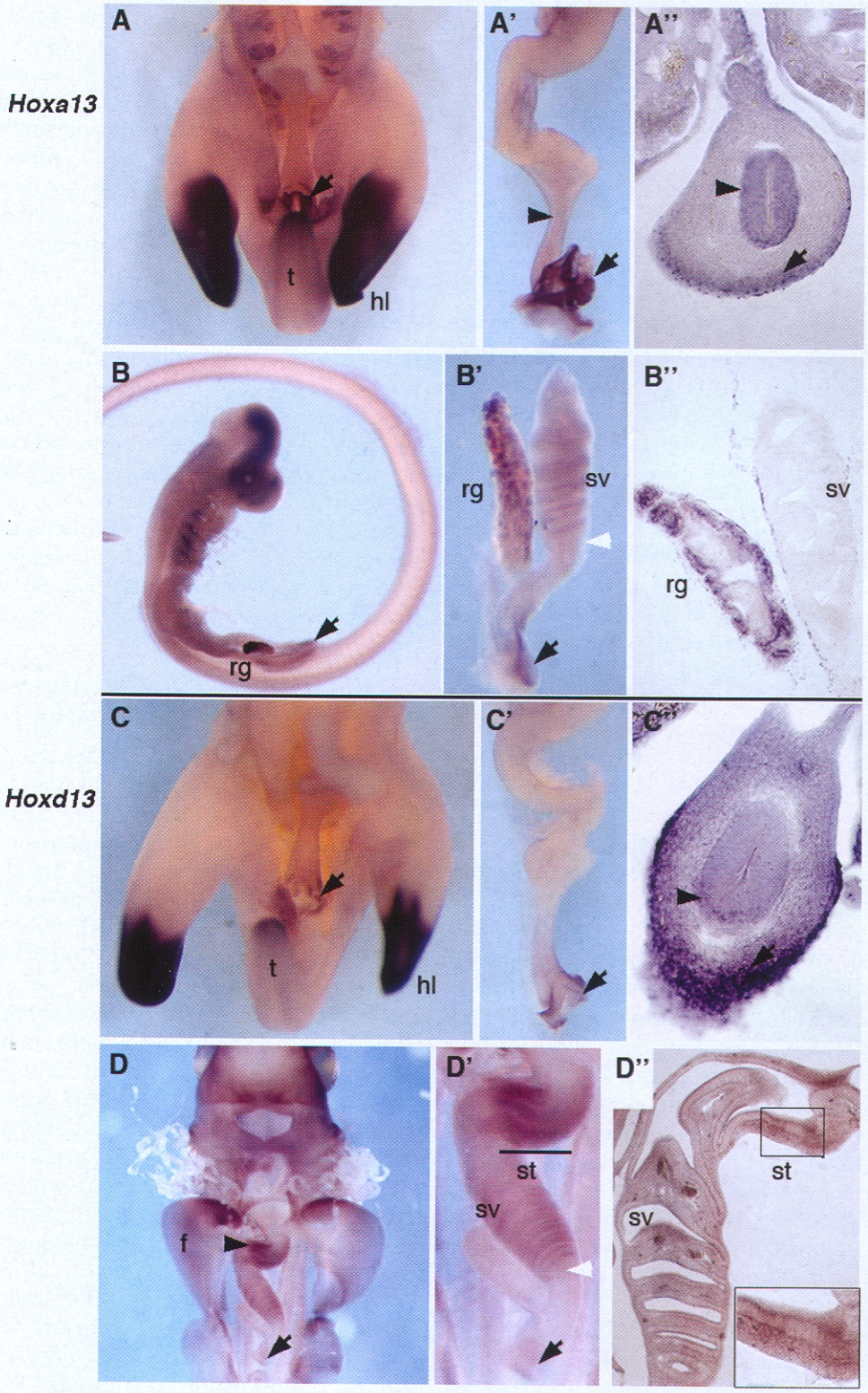
Hoxa13 and Hoxd13 expression patterns in R. erinacea

Hoxa13 and *Hoxd13* pattern the colon in chick and mouse (Kondo et al., '96; Warot et al., '97; Roberts et al., '98; de-Santa-Barbara and Roberts, 2002). In the chick hindgut, both *Hoxa13* and *Hoxd13* are expressed in the cloaca (Fig. 5A and C). *Hoxa13* is expressed in both the cloaca mesoderm and endoderm, with endoderm expression extending anterior into the ceca (Fig. 5A' and A''). In contrast, *Hoxd13* transcripts are present in the cloaca mesoderm, with only faint endoderm staining at the cloaca itself (Fig. 5C' and C''). Further expression of *Hoxd13* in the hindgut endoderm is not observed (data not shown). As the viral misexpression of *Hoxd13* in the gut mesoderm has been directly linked to the presence of acid mucins in the endoderm, we examined the expression patterns of *Hoxa13* and *Hoxd13* in *R. erinacea* embryos (Roberts et al., '98). As in chick, both *Hoxa13* and *Hoxd13* are expressed in the cloaca (Fig. 5B' and D). Additional expression of *Hoxa13* is observed in the rectal gland mesoderm, an organ that regulates ionic levels in the blood of elasmobranches (Fig. 5B''). *Hoxa13* is not expressed in the hindgut endoderm of little skate as seen in chick (data not shown). In addition to the cloaca, whole mounts for *Hoxd13* transcripts display strong expression in the posterior stomach and faint expression in the spiral valve intestine (Fig. 5D and D'). Faint expression of *Hoxd13* in the spiral valve intestine is not distinguishable above background on sections (Fig. 5D'').

Fig. 5. *Hoxa13* and *Hoxd13* expression patterns in chick and little skate embryos. Whole mount RNA in situ hybridization of transcripts *Hoxa13* and *Hoxd13* in chick (**A**, **A'**, **C**, **C'**) and little skate (**B**, **B'**, **D**, **D'**) embryos. (A) *Hoxa13* gene expression observed in the tip of the tail, the hindlimbs and the cloaca (arrow). (A') The digestive tract removed in (A) to better illustrate cloaca expression (arrow) and faint expression in hindgut endoderm (arrowhead). (A'') Expression of *Hoxa13* in the endoderm (arrowhead) and mesoderm (arrow) is distinct on a transverse section at the hindgut-cloaca border. (B) *Hoxa13* expression in the little skate rectal gland and cloaca (arrow). (B') Digestive tract in (B) shows strong expression in the rectal gland and cloaca (black arrow), and no staining in spiral valve coil 8 (white arrowhead; sv8 in Fig. 3A). (B'') Sagittal section of an in situ for *Hoxa13* demonstrates strong expression in the mesoderm of the rectal gland and no expression in the spiral valve intestine. (C, C') *Hoxd13* is expressed in the tail tip, hindlimbs and cloaca (arrows). (C'') Transverse section through the cloaca highlights strong *Hoxd13* mesoderm expression (arrow) with some faint endoderm expression (arrowhead). No *Hoxd13* transcript was detected in more anterior regions on of hindgut with sections. (D) In the little skate, *Hoxd13* expression is found in the lateral fin buds, stomach (arrowhead) and cloaca (arrow). (D') High magnification of the digestive tract in (D) highlights *Hoxd13* expression in the stomach (black line), cloaca (arrow) and faintly in the spiral valve mesoderm. *Hoxd13* is absent from spiral valve coil number 8 (white arrowhead). (D'') Merged images of sagittal sections through the stomach and spiral intestine; t, tail. Stomach expression for *Hoxd13* verified by sagittal section (inset). f, fin; hl, hindlimb; rg, rectal gland; sv, spiral valve intestine; t, tail.

DISCUSSION

In this study, we set out to characterize the spiral valve intestine and large intestine of *R. erinacea* and begin to understand if patterning mechanisms of *Hox* genes in the hindgut are conserved in evolution of the vertebrate digestive tract.



R. erinacea lacks a functional colon

Studies concerning the elasmobranch digestive tract have encompassed basic morphological structures; the spiral valve, truncated colon, rectum, and cloaca have well documented histologies (Romer and Parsons, '85; Lacy, '91; Stevens and Hume, '95; Chatchavalvanich et al., 2006). This study examines the presence of mucosubstrates in the intestines to consider the functions of different regions of the chondrichthian midgut and hindgut. In stage 33 *R. erinacea* embryos, the anterior six coils of the spiral valve are lined with villi on all surfaces and contain low numbers of acid mucin-producing cells (1–4%) (Fig. 3A and B). In coils 7 and 8, where the villi are replaced by a glandular columnar epithelium, the percentage of cells producing acid mucins rise to 19% (Figs. 2 and 3C). In the squamous epithelium of the little skate hindgut, the percentage of acid mucin-producing cells dropped to basal levels found in the anterior spiral valve (3%) (Fig. 3D). In the hatched animal, an overall increase in acid mucins was observed, most likely due to the colonization of the intestines by bacteria introduced from the marine environment during ingestion (Fig. 2) (Filipe, '79; Corfield et al., '92; Lugea et al., 2000; Backhed et al., 2005). Strikingly, although the overall percentages increased, the overall distribution of acid mucins remained similar to that found in the embryo, with the highest percentage of acid mucin cells in the final posterior coils 7 and 8 of the spiral valve (33 and 42%, respectively), and levels of the colon matching those of the anterior spiral valve (21 and 20%, respectively) (Fig. 2). These results suggest the embryonic pattern of cell distributions is maintained post-hatching despite the introduction of colonizing bacteria in the digestive tract, further suggesting that the function of the sub-regions of the intestines is the same across development.

The specific distribution of mucins throughout the gut allows for the efficiency of digestion, protection of the epithelial lining from sheering with the passage of food, and supports the existence of microflora, partly as a result of maintenance of certain pH (Filipe, '79; Lichtenberger, '95; Corfield et al., 2000; Lugea et al., 2000; Roussel and Delmotte, 2004). Our results from *R. erinacea* differ from distributions found in terrestrial vertebrates. In E17.5 mouse embryos, we observe low levels (1.5%) of acid mucins produced in the small intestine with increased levels in the large intestine epithelium (24%) (Fig. 4A and B). Our results for the mouse correlate with levels observed in other land vertebrates such as human and chicken (Filipe, '79; Roberts et al., '98). The human small intestine exhibits a low presence of acidic mucins in the small intestine, and levels rise towards the posterior regions where the small intestine transitions into the colon (Filipe, '79) (Buisine et al., '98). Similarly, 6% of cells in the chick embryo small intestine produce acid mucins, compared to 33% in the colon (Roberts et al., '98). Although the hindgut region of *R. erinacea* contains simple squamous epithelial cells as does the hindgut of other vertebrate species, the low numbers of acid mucins observed in this region confirm that the hindgut region of the elasmobranch does not function as a water absorption organ as it does in higher vertebrates such as chick, mouse and human (Fig. 4C) (Filipe, '79; Lacy, '91; Buisine et al., '98; Roberts et al., '98; Lugea et al., 2000). The hindgut is responsible for absorption of water against a high osmotic gradient in most vertebrates and serves to maximize the water re-absorption from an organism's feces (Holmes and Donaldson, '69; Randall et al., '97). Despite the similar cellular morphology, the unique osmoregulatory and ionoregulatory properties of the elasmobranch support the hypothesis that these fish do not possess a functional colon.

Evidence of a rudimentary colon in the posterior spiral valve intestine

The evolution of the colon contributed greatly to the development of terrestrial life. The absorption of water from waste products is one of several mechanisms crucial for the maintenance of water levels in land vertebrates. The blood osmolarity of elasmobranchs is slightly hyperosmotic to ocean water, which is maintained through the retention of urea and TMAO (Holmes and Donaldson, '69). Because the gills are fairly permeable, water diffuses through the gills (Stevens and Hume, '95). The ability of the elasmobranchs to maintain a high cellular osmolarity without increasing the presence of ions suggests a similarly unique function of the colon. In contrast, teleosts suffer diffusive water losses to the environment through their gills and must take in water through the digestive tract, thus their cell osmotic content is slightly lower than the marine environment (Smith, '36; Reifel and Travill, '79).

The terrestrial vertebrates and the elasmobranchs have markedly different distributions of acid mucins, reflecting the varying osmotic regulation in the different animals. The presence of

mucins at 19%/42% (embryonic/post-embryonic) in the final turn of the spiral valve and the subsequent return to lower levels seen in the colon (3%/21%) supports the idea that the elasmobranch colon does not serve as a water re-uptake organ in cartilaginous fish as it does in land vertebrates. The elevated acid mucins in the final turn of the spiral valve correspond with a change to a more glandular columnar epithelial morphology in the spiral intestine, suggesting an evolutionary pattern for the development of the colon in higher vertebrates. The distribution of mucins in the little skate colon relative to that of the mouse could suggest three possible models for the evolution of the colon. The final turn of the spiral valve intestine containing acid mucins may itself be a water re-uptake organ outside of the hindgut. This model could be tested by examining for the presence of aquaporin-type water channels in the posterior spiral intestine (Ma and Verkman, '99). Another possibility is that an ancestor to *R. erinacea* may have possessed a colon similar to that of the chick, containing high levels of acid mucins. The Chondrichthyes may have lost the function of the colon and thus the presence of acid mucins. This could account for the presence of elevated acid mucins in the last turn of the spiral valve, as it may be a remnant of the ancestral colon. In light of the iso-tonic nature of the more primitive vertebrates (the hagfish), it seems likely that the terrestrial colon developed from an expansion of the eighth turn of the spiral valve or a similar structure shared by a common ancestor to the cartilaginous and bony fishes (Randall et al., '97; Muller et al., 2003). Because the hagfish do not require water regulation mechanisms as a result of their cellular osmolarity, it is reasonable that they would not require a functional colon. Further analysis of the distribution of acid mucins in the most primitive craniates (the osmoconformer hagfish that lacks a spiral valve, and the osmoregulator lamprey that contains a spiral valve) is needed to further test this theory for the origin of the colon.

Rudimentary colon in R. erinacea may be patterned by Hoxa13 and Hoxd13

The posterior *Hox* genes, *Hoxa13* and *Hoxd13* have been demonstrated to pattern the hindgut in chick, mouse and humans (Kondo et al., '96; Warot et al., '97; Roberts et al., '98; Goodman and Scambler, 2001; de-Santa-Barbara and Roberts, 2002). Both *Hoxa13* and *Hoxd13* are expressed in the cloaca and rectum of chick and mouse, respectively, with some additional expression of *Hoxa13* in the chick hindgut endoderm (Fig. 5A'' and C'') (Warot et al., '97; Roberts et al., '98). Removal of functional copies of these genes results in sphincter and rectal malformations (Kondo et al., '96; Warot et al., '97; Goodman and Scambler, 2001). In addition, viral misexpression of *Hoxd13* in the midgut mesoderm in chick leads to a transformation of the small intestine epithelium to a colon-like morphology with elevated acid mucins levels (Roberts et al., '98). Although *Hoxd13* in the chick has been demonstrated to pattern the presence of acid mucins in the chick colon, *Hoxd13* expression is not observed in the hindgut mesoderm or endoderm (Fig. 5C and C'') (Roberts et al., '98). Thus, the mechanism in which *Hoxd13* patterns the colon remains unknown.

We investigated whether *Hoxa13* and *Hoxd13* pattern the little skate hindgut despite the lack of a functional colon. As seen in chick, *Hoxa13* and *Hoxd13* expression is present in the cloaca of *R. erinacea*. While *Hoxa13* is expressed in the chick hindgut endoderm, *Hoxa13* expression in little skate is only found in the cloaca and not in hindgut sections (Fig. 5A' and B' and data not shown). As the expression patterns for *Hoxa13* and *Hoxd13* are conserved in the cloaca of chick and little skate, and cloacal expression has been linked to patterning of the chick hindgut (Roberts et al., '98), it remains a possibility that *Hoxd13* may function in patterning the acid mucins found in the posterior spiral valve intestine. Further work is needed to examine gene function for *Hoxd13* in the developing little skate, as well as elucidate the mechanism by which *Hoxd13* is able to pattern acid mucins in the terrestrial colon.

Interestingly, *Hoxd13* expression in little skate deviated from the chick in the strong domain of expression in the posterior stomach (Fig. 5D'). *Hoxd13* expression has not been found as anterior as the stomach in any other vertebrate to date, suggesting that the regulation of *Hoxd13* expression in *R. erinacea* is by a yet to be described mechanism. Changes in non-coding sequences within the *Hox* gene cluster has arisen from duplication of the cluster during evolution of the vertebrate lineage (Holland et al., '94; Malaga-Trillo and Meyer, 2001; Wagner et al., 2003; Prohaska and Stadler, 2004). These changes may lead to changes in *Hox* gene expression patterns, and ultimately gross changes in body plan can result from altered transcriptional regulation of

specific *Hox* genes (Martinez and Amemiya, 2002). A loss of conservation in cis-regulatory regions has been observed for the 5' (or posterior) region of the *Hox* cluster in Chondrichyes further supporting that the altered *Hoxd13* expression pattern in the *R. erinacea* stomach may be the result of altered *Hox* gene patterning (Chiu et al., 2002).

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