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Elucidating a role for Hoxd13 during gut development in the chick and skate

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Elucidating a role for *Hoxd13* during gut development in the chick and skate

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ABSTRACT

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The *Hox* genes are a family of highly conserved transcription factors that are critical during embryonic development. Although the function of *Hox* genes is to pattern the embryo from head to tail, the mechanism by which *Hox* genes do this is unknown. Elucidating the mechanism for *Hox* gene function can provide insights into how morphologies are formed in a variety of organisms. I am investigating the role of *Hoxd13* in patterning the colon in the developing chick. We virally misexpressed the chick ortholog, *cHoxd13*, in the midgut mesoderm of the chick to validate that transformation to hindgut morphology would occur. To determine if the role of *Hoxd13* has been conserved since the evolution of the cartilaginous fish, we will misexpress the skate ortholog of the gene, *LeHoxd13*, in the chick embryo and check for transformation to colon morphology. To do this, we are constructing a retrovirus that will express the skate *Hoxd13* gene in the chick embryo. If we can show that hindgut character is induced in the midgut with viral misexpression of *cHoxd13* and *LeHoxd13*, our hypotheses that *Hoxd13* serves to pattern the embryonic colon, and that its function is evolutionarily conserved, will be supported.

Introduction

A comprehensive understanding of embryonic gut patterning is essential to obtain an evolutionary perspective of both the anatomy and physiological function of today's vertebrate colon. Gut patterning in the early embryo allows for the primitive gut tube to differentiate into the specialized organs of the digestive tract, each with unique and complex functions. Among these specialized organs is the colon. The colon is critical for survival of vertebrates on land, as it absorbs water from the feces. However, little is known about the evolution of this organ. Studies have shown that the *Hox* homeobox gene family, specifically the gene *Hoxd13*, plays a significant role in patterning the gut tube. Elucidating *Hoxd13* gene function will provide insight into the mechanisms governing development of the colon, and can also contribute significantly to the general understanding of *Hox* gene function within embryonic development. Further, knowledge of both colon development and *Hox* gene function may have valuable application in the pathology of several colon-related diseases, cancers and anorectal malformations for which targeted treatments have not yet been developed.

The primary functions of the colon in vertebrates include water and electrolyte absorption, storage of waste, fermentation of carbohydrates and proteins, and defecation (Bijlani, 2004). The proximal portion of the colon is primarily responsible for water and electrolyte absorption, which are facilitated by the relatively permeable epithelium of this section of the gut tube. In the proximal colon, sodium is actively absorbed, while chloride, potassium and water are all passively absorbed (Bijlani, 2004). The colon is highly efficient in absorption, as it absorbs approximately 99% of the water which enters

it (Bijlani, 2004). Bacteria residing in the lumen and mucosa of the colon aid in fermenting carbohydrates and proteins. During fermentation, undigested proteins, carbohydrates, fats and dietary fiber are degraded, thus allowing the resulting nutrients to be absorbed. The colon also controls motility of its contents; it propels the feces gently toward the rectum (Bijlani, 2004).

The absorptive ability of the colon plays a critical role in maintaining an appropriate water balance within land-dwelling vertebrates. The colon is assumed to have evolved as animals migrated out of the water and onto land approximately 375 million years ago (Thanukos, 2008). In this evolution, water retention was a primary challenge. Animals evolving from sea to land also required a way to balance concentrations of solutes in the body (Lacy, 1991). The evolution of specialized epithelium in the colon provided a mechanism for both solute and water balance (Lacy, 1991). Furthermore, the colon's evolved capacity to store waste was advantageous as it allowed the organism to deposit feces in specific locations instead of leaving a continuous trail which could be easily detected by predators (Lacy, 1991).

The colon is histologically distinct from other organs of the digestive tract. While the small intestine epithelium contains villi, which dramatically increase absorptive surface area, the colon epithelium contains flattened villi. Villi are flat within the colon because deep crypts within the lumen would hinder the movement of feces along the intestinal tract (Roberts et al., 1998; de Santa Barbara et al., 2003). The colon is also characterized by the presence of acid mucin-producing cells called goblet cells, which are interspersed among intestinal absorptive cells in the gut tube lumen (Roberts et al., 1998). Goblet cells in the colon produce acidic mucins, while in the small intestine, they produce

neutral mucins. The mucins serve to lubricate the lumen and protect the gut from direct contact with food particles (Sakata & Engelhardt, 1981). In the colon, acidic mucins facilitate water absorption (Theodosiou et al., 2007; Roberts et al., 1998).

The colon's distinct histology arises from specific molecular signaling between germ layers during formation of the gut tube. The colon, which is part of the lower gastrointestinal (GI) tract, originates from a gut tube composed of two layers of cells derived from the endoderm and mesoderm. The initial gut tube forms from two invaginations in the primitive endoderm located at each end of the embryo. The invagiantion at the anterior end of the embryo is known as the anterior intestinal portal (AIP), and the invagination at the posterior end is the caudal intestinal portal (CIP). At the AIP, mesoderm surrounding the invaginated endoderm differentiates into smooth muscle and forms a tube around the endoderm (Sanderson & Walker, 2000). This tube develops as an open cylinder and runs along the anterior-posterior (AP) body axis. Mesoderm at the posterior end of the embryo envelops the endoderm of the CIP in a way similar to anterior tube formation. The posterior end of the tube develops as an open cylinder and elongates toward the AIP-derived tube. These tubes formed from the AIP and CIP meet and fuse in the center of the embryo at the umbilicus (Sanderson & Walker, 2000). The single fused tube later becomes subdivided into three regions known as the foregut, hindgut and midgut, which are derived from AIP cells, CIP cells and a combination of AIP and CIP cells, respectively. The foregut will give rise to the esophagus, the stomach and part of the duodenum of the small intestine, as well as the liver and pancreas. The midgut gives rise to the remaining parts of the duodenum and the small intestine, the appendix, the cecum, the ascending colon and 2/3 of the transverse

colon. Finally, the hindgut differentiates into the remainder of the transverse colon, the rectum and the anal canal (Conner & Dawson, 2003). Communication between cells along the AP body axis helps to direct organ development in the proper order. To insure the correct function of organs, the development and differentiation of tissue layers require substantial communication between the endoderm and mesoderm. One such signal is *Sonic hedgehog* (*Shh*).

Shh, a ligand for Hedgehog signaling, is expressed in both the AIP and the CIP. Studies in the chick show that as the gut tube forms and differentiates, *Shh* expression spreads throughout the gut endoderm (de Santa Barbara et al., 2003). The *Shh* signal functions by directing cell proliferation in the overlying mesoderm. In the mesoderm, *Shh* turns on different downstream target genes to specify the location of different organs (Roberts et al., 1998). A downstream target of *Shh* in the mesoderm is the *Hox* family of transcription factors (Roberts et al., 1998).

The *Hox* genes encode a family of transcription factors that pattern the body axis of the embryo. The expression domains of *Hox* genes correspond with their roles in specifying the body axis and the organ systems along the entire AP axis of the embryo (Huang et al., 1998). Along with specifying the body axis, normal expression of *Hox* genes is critical for formation and development of the gut tube. *Hoxd13* plays a role in specifying hindgut morphology. *Hoxd13* is expressed in the hindgut of the developing mouse and chick embryos. Loss-of-function *Hoxd13* mice have abnormal sphincter muscles and anterior character in the hindgut, further supporting that *Hoxd13* is involved in stimulating posterior morphology in the hindgut (Kondo et al., 1996; Warot et al., 1997).

During chick development, *Hoxd13* is expressed in the hindgut mesoderm and endoderm unlike other *Hox* genes that are expressed exclusively in the mesoderm. Misexpression of *Shh* in the embryonic chick mesoderm leads to ectopic expression of *Hoxa13* and *Hoxd13,* indicating that these genes are downstream targets of *Shh* (Roberts et al., 1995). Furthermore, viral-misexpression of *Hoxd13* in the mesoderm of the midgut induces hindgut morphology in the midgut endoderm, suggesting a critical role for *Hoxd13* in directing differentiation of the hindgut during development (Roberts et al., 1998). Additionally, the presence of acid mucins and flattened villi in the small intestine, both indicative of colon endoderm, have been observed in the endoderm tissue adjacent to the *Hoxd13*-misexpressed mesoderm (Roberts et al., 1998). Though progress has been made in elucidating the initial role for *Hoxd13* function, the evolutionary history of this gene remains unknown. The contribution of *Hoxd13* to the development of a fully-functioning colon suggests that its role may be conserved among land animals, however, its presence in earlier animals has not yet been determined.

The aim of this work is to determine whether the function of *Hoxd13* is conserved among vertebrates. This knowledge will provide insight into the evolution of our own species, and specifically into the molecular mechanisms underlying vertebrate gut development. I examined viral misexpression of the chick *Hoxd13* ortholog, *cHoxd13,* in the chick. Based on previous work, *cHoxd13* should transform the midgut endoderm to a hindgut morphology. This will confirm that *cHoxd13* can specify the fate of the midgut endoderm cells to a hindgut character. We will be able to confirm that hindgut transformation has occurred in the midgut endoderm if wide, flat villi are present and if an increase in the number of cells producing acid mucins is detected. We further predict

that the function of *Hoxd13* is conserved among vertebrates. The skate, *Leucoraja erinacea*, is member of the Chondrichthyes class of cartilaginous fish. If the skate *Hoxd13* ortholog, *LeHoxd13*, can induce hindgut morphology in the midgut endoderm, we can deduce that the function of *Hoxd13* has been conserved since evolution of the Chondrichthyes. We will test this hypothesis by injecting the skate ortholog, *LeHoxd13*, into the midgut mesoderm of the chick and determining whether the same transformation to hindgut morphology occurs in the midgut endoderm.

Materials and Methods

Embryos

Fertilized chick eggs were obtained from the University of Connecticut Poultry Farm (Storrs, CT). Eggs were incubated at 39°C and staged according to day of incubation (e, embryonic day).

Viral Misexpression

Two day chick embryos were injected with a replication-competent retroviral vector with alkaline phosphate (RCAS-AP). A window was cut into each egg shell to expose the embryo, and embryos were viewed using a Leica MZ6 microscope. Virus was injected into the intercoelomic space of the chick embryo, targeting viral infection to the gut mesoderm. The window was sealed with tape and embryos were placed in an incubator at 39°C. Embryos were incubated until embryonic day 9 (e9) and harvested. The gut tube was dissected out of each embryo, fixed in 4% paraformaldehyde/PBS,

dehydrated to 100% methanol and stored at -20°C. For e9 embryos, whole mount *in situ* hybridization was performed with a RNA probe against the viral *gag* gene to confirm viral infection (Figure 1) (Riddle et al., 1993; Burke et al., 1995). To misexpress *cHoxd13*, chick embryos were infected with an RCAS-AP-*cHoxd13* virus (Hughes et al., 1987). Eggs were incubated to e18, harvested, fixed in 4% paraformaldehyde/PBS and paraffin embedded for sectioning (Allen, 1994; Murtaugh et al., 1999).

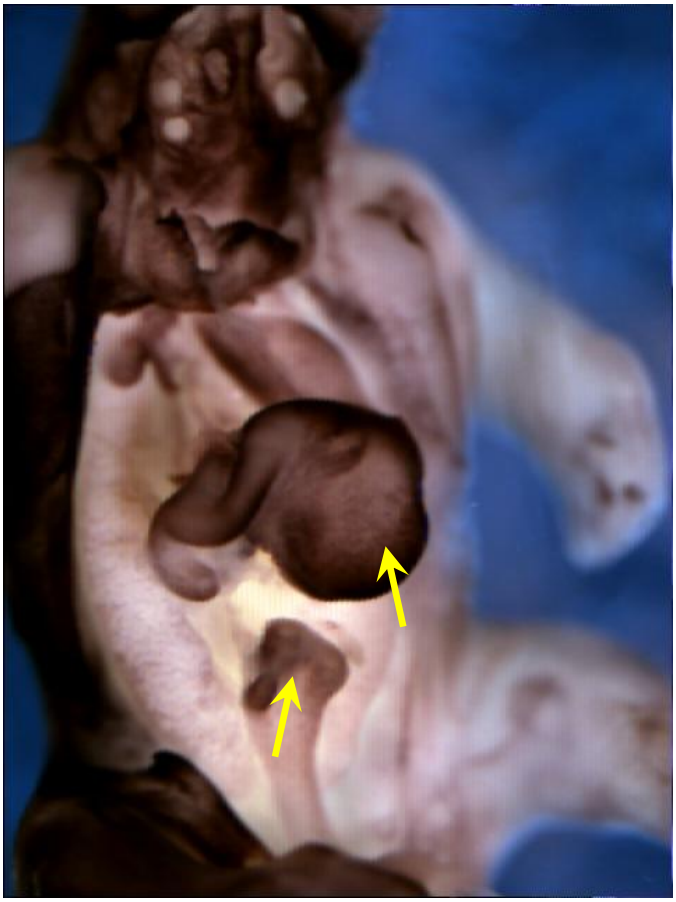

Fig. 1. Viral misexpression patterns in chick embryo at day 18 of embryonic development shown with whole mount RNA in situ hybridization of viral *gag* transcript. Viral expression is targeted to the gizzard (arrow) and hindgut (arrow).

Histology and Immunochemistry

Seven RCAS-*cHoxd13*-infected guts and two wild type control guts were cut into 8μ sections and mounted onto slides. Adjacent sections of tissue were placed on sequential slides to facilitate comparison of viral infection with gut histology. Alternating slides were stained using Hematoxylin and Eosin, Nuclear Fast Red and Alcian Blue, or the 3C2 antibody, which detected viral infection against the *gag* viral protein and was visualized by DAB staining (Allen, 1994; Theodosiou & Tabin 2005). Hematoxylin and Eosin stained cell nuclei blue and the majority of the cytoplasm in shades of red and pink. The Nuclear Fast Red and Alcian Blue stain identified acid mucins in the tissue blue, and cell nuclei pink. The 3C2 antibody detected areas of viral infection in the gut tissue.

To stain for acid mucins with Nuclear Fast Red and Alcian Blue, slides were de-parrafinized in xylene and hydrated in a descending alcohol series. Slides were rinsed in dH_2O, stained in Alcian Blue Solution at pH 2.5 for 5 minutes and rinsed in running tap water for 3 minutes. To identify nuclei, tissue was stained in Nuclear Fast Red for 10 minutes and rinsed in running tap water for 3 minutes (Electron Miscoscopy Sciences, Fort Washington, PA). Slides were rinsed in distilled water, dehydrated in an ascending alcohol series followed by xylene, and mounted with DPX permanent mountant. The presence of acid mucins was observed using a Nikon Eclipse E600 microscope.

Cloning *L. erinacea Hoxd13* into RCAS for misexpression

In order to examine how conserved the role of the skate *Hoxd13* is during gut development, we cloned the skate *LeHoxd13* into an RCAS viral vector for

misexpression in the chick. The full length *LeHoxd13* gene in our lab contained an intron. In order to do misexpression studies, it was necessary to remove the intron before subcloning the gene into the RCAS vector. To remove the intron from the full length *LeHoxd13* gene, the 5' end (600bp) of the gene was isolated by cutting with XbaI and SmaI, gel isolated, and cloned into Bluescript vector. Next, the 3' end (254bp) of *LeHoxd13* was isolated by PCR amplification using primers (5' primer AAAAGTACTTTCAGGAGATGTGATGC and 3' primer CGGAATTCCGTCAAGGTAT). The primers used for PCR contained both Sca and EcoR1 sites engineered in at the 5' and 3' ends, respectively. The Bluescript vector containing the 5' fragment was cut with Sma1 and EcoR1 and the 3' end was ligated in, as Sma1 and Sca1 have compatible ends. The final product was confirmed by sequencing.

To subclone *LeHoxd13* into the RCAS vector, *BS-LeHoxd13* was cut with NcoI and EcoR1 to isolate the full length gene. *LeHoxd13* was inserted into the *pSlax* vector. *pSlax-LeHoxd13* was cut with Cla1, isolating the full length gene and inframe promoter from *pSlax*, and was ligated into the *RCAS* vector cut with Cla1. The final RCAS-*LeHoxd13* product was confirmed by sequencing.

Results

Previous research indicates that the function of *Hoxd13* is to induce hindgut morphology in the developing embryo (Roberts et al., 1998). To further investigate the

role of *cHoxd13* in specification of the colon, we analyzed the gut tube morphologies of seven embryos injected with *cHoxd13* in the intercoelomic space of the embryo, targeting viral infection in the gut splanchnic mesoderm. Infected gut morphologies were compared with two wildtype control guts. The *cHoxd13*-infected gut tubes appeared shorter in length and contained less looping than the wildtype (Figure 2). There was also more bile present in the *cHoxd13*-infected embryo gut tube, indicated by green color inside the gut tube. We noted visible variation from the wildtype phenotype in four of the seven guts analyzed. The remaining three were considered to have no detectable phenotype in terms of their gross morphologies**.**

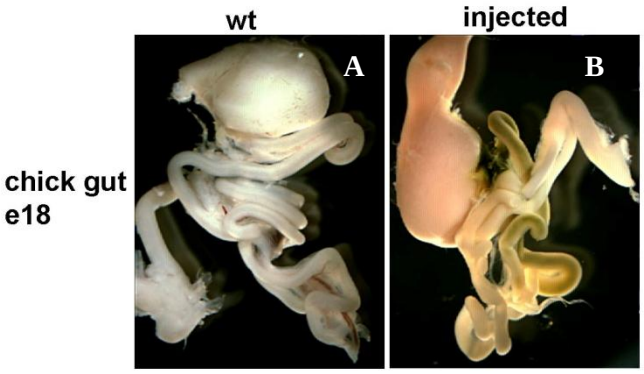

Fig. 2. Comparison of wildtype and *cHoxd13*-injected digestive tracts dissected from embryonic day 18 chick embryos.. (A) Wildtype digestive tract compared to (B) *Hoxd13*-injected digestive tract. Digestive tracts misexpressing *Hoxd13* appear shorter than wildtype and contain less looping. Greenish bile is also present in the injected digestive tract.

To better discern the misexpression phenotype, *cHoxd13*-infected guts were analyzed by histology. Phenotypes were observed in the gizzard, small intestine and ceca. Phenotypes included changes in epithelial morphology, disorganization of the submucosa layer and abnormal structures within the submucosa.

Analysis of the small intestine section of the gut identified several abnormal phenotypes, all of which corresponded with viral infection as indicated by 3C2 staining (Figure 3). Two phenotypes were observed in the epithelium. Some *cHoxd13*-infected guts possessed large, globular-shaped villi containing high counts of red blood cells (n=3). Some guts showed both this phenotype, as well as sections of epithelium containing flat, blunted villi closely resembling those found in the wildtype hindgut (n=2).

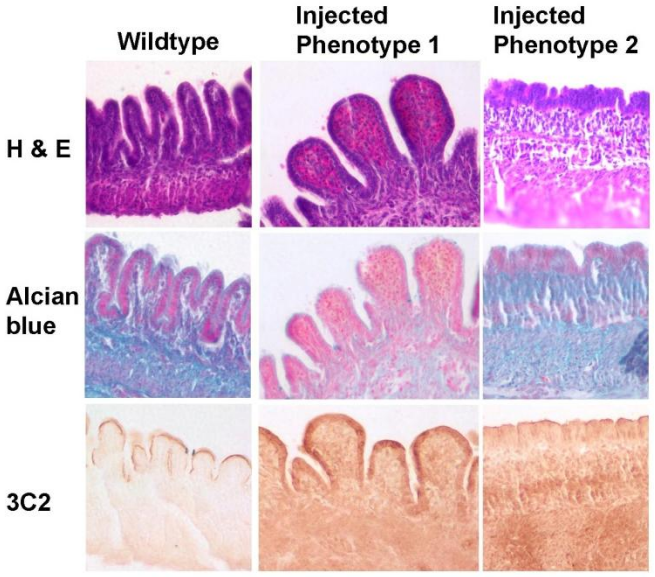

Fig. 3. Histological stains of small intestine tissue with c*Hoxd13* misexpression showing two phenotypes: short, blunted villi resembling hindgut epithelium, and large, bulbous villi. Morphology visualized with histological stains Hemotoxylin/Eosin, Alcian Blue/Nuclear Fast Red and 3C2. Eight micron sections through the small intestine of a chick embryo at day 18 of embryonic development. Phenotype 1 shows bulbous villi containing high counts of red blood cells and strong viral infection (3C2). Phenotype 2 shows flattened villi resembling wildtype hindgut morphology.

In the gizzard, large vacuole-like structures were observed in the endoderm and into the submucosa (n=4) (Figure 4). The submucosa appeared disorganized and indistinguishable from the epithelial cell layer (n=2). Epithelium appeared wildtype in

some guts (n=2) and slightly flattened in another (n=1). 3C2 staining showed prevalent viral infection in the mesoderm and submucosa in the corresponding gizzard sections (n=3).

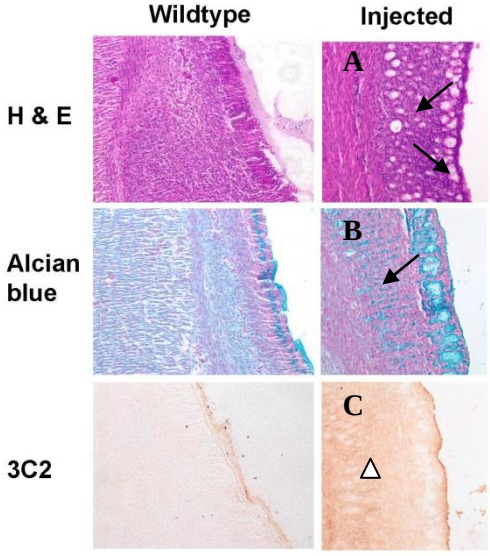

Fig. 4. Histological stains of gizzard tissue containing *cHoxd13* misexpression showing large vacuoles and disorganization in the submucosa. Morphology visualized with Hemotoxylin/Eosin, Alcian Blue/Nuclear Fast Red and 3C2 stains. Eight micron sections through the gizzard of a chick embryo at day 18 of embryonic development. (A, B) Large vacuoles were observed in the submucosal layer (arrows). Highly disorganized submucosa was observed (arrowhead, B). (C) Viral infection strong throughout submucosa .

Analysis of the ceca sections showed villi of irregular shape (Figure 5). Villi appearances were not consistent; some long, bulbous shapes were observed (n=1) as well as short, blunted villi (n=2). Both the bulbous and flattened villi phenotypes also appeared within the same gut (n=1). The germ layers within the ceca appeared less distinct (n=3), and the submucosal layer was disorganized with respect to cell form (n=1). Strong viral infection was observed in the submucosal layer, corresponding with the abnormal phenotypes (n=3).

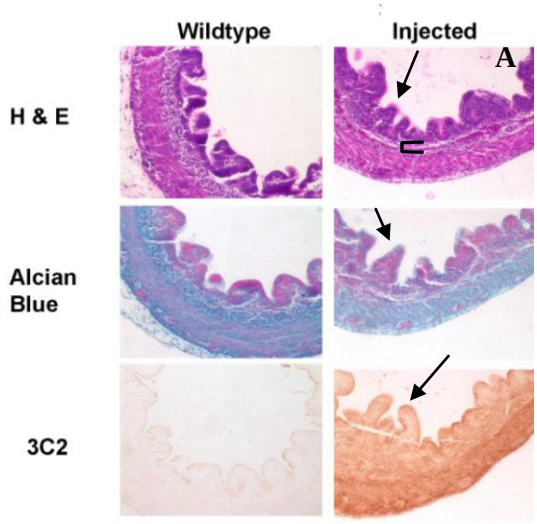

Fig. 5. Histological stains of ceca tissue from embryo containing viral misexpression of *cHoxd13* showing abnormal epithelium and thinned submucosa. Ceca morphology visualized with Hemotoxylin/Eosin, Alcian Blue/Nuclear Fast Red and 3C2 stains. Eight micron sections through the ceca of a chick embryo at day 18 of embryonic development. Inconsistent and abnormal buckling of the epithelium (arrows) and thinned submucosa layer (A) observed in the injected gut.

In order to test the evolutionary conservation of *Hoxd13* patterning, we wanted to build a construct to misexpress the skate ortholog of *Hoxd13* in the chick. The skate ortholog, *LeHoxd13*, was successfully inserted into an RCAS viral vector. The intron present in the original full length gene was excised and the 3'and 5' end of the gene were rejoined and ligated into the bacterial plasmid vector, Bluescript. The gene was cut using restriction enzymes and placed into a shuttle vector, *pSlax*, containing the viral promoter region. The gene was cut a final time and ligated into the RCAS viral vector. To confirm the gene was inserted into RCAS, the construct was cut with Cla1 and by gel electrophoresis, a band of 150 bp was resolved (Figure 6). The full viral vector was sequenced to confirm that *LeHoxd13* was successfully inserted.

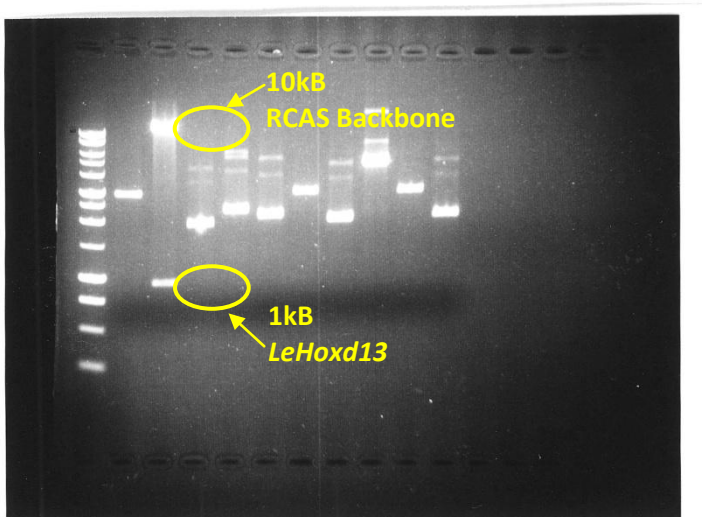

Fig. 6. SDS-PAGE gel electrophoresis of the RCAS viral vector containing the skate *LeHoxd13*. In lane 3, the band at 10kB represents the RCAS backbone. The band at 1kB is the gene *LeHoxd13* with the 150kB intron removed. This gel confirms that *LeHoxd13* was successfully inserted into the RCAS viral vector.

Discussion

In this study I aimed to elucidate the role of *Hoxd13* in the developing chick embryo, and to determine if *Hoxd13* gene function has been conserved since the evolution of the cartilaginous fish. Misexpression of c*Hoxd13* by retroviral infection in the midgut of the developing chick leads to several phenotypes, including some previously reported by Roberts et al. (1998).

Chick *Hoxd13* misexpression leads to repatterning of the gut endoderm

Deviation from the wildtype chick endoderm was observed in the gizzard, small intestine and ceca. The results of this experiment confirmed the phenotypes observed by

Roberts et al. (1998) in the small intestine, but also recognized additional phenotypes not noted in previous literature, including in the small intestine, gizzard and ceca.

It has been reported that viral misexpression of c*Hoxd13* in the mesoderm of the chick embryo midgut results in transformation to hindgut morphology and physiology within the endoderm adjacent to the injected mesoderm (Roberts et al., 1998). In previous research, flat, blunted villi, sporadic glands and an acid mucin pattern characteristic of the hindgut were observed within the epithelium of the *cHoxd13*-infected midgut in the chick. We also identified flattened villi in the small intestine resembling hindgut morphology with ectopic expression of *cHoxd13*. Unlike previous studies, however, we observed additional phenotypes in the small intestine and in the gizzard and ceca, which included irregularly shaped epithelium and abnormally indistinct boundaries between tissue layers. Variation from the wildtype with respect to the length of the digestive tract components was also noted in contrast to previous findings.

In the gizzard, the epithelial layer of the *cHoxd13*-injected embryos was notably thinner than in the wildtype embryos. The virus-infected epithelium also appeared smoother than wildtype epithelium. These results suggest that the *cHoxd13* virus affected the normal patterning scheme within tissues and may be responsible for determining morphologies of the different germ layers. Viral infection was most prominent within the muscle and submucosa layers and faint in the epithelium. Since a distinct phenotype was observed within the epithelium despite minimal viral infection, it is suggested that the viral effects are mediated by signaling pathways between the germ layers.

Analysis of the *cHoxd13* virus-infected small intestine endoderm identified two distinct phenotypes. The first phenotype corresponded with the results described in Roberts et al. (1998), and consisted of broad, flattened villi. These results imply conversion of the small intestine to hindgut morphology with misexpression of *Hoxd13*. The second phenotype observed in the small intestine endoderm consisted of large, globularly shaped villi on the epithelium. These villi contained large amounts of red blood cells and capillaries normally found only in the adjacent submucosal layer. This phenotype was not reported by Roberts et al. (1998), and represents a stark contrast to the first phenotype observed. Both the flattened and globular villi phenotypes corresponded to areas of substantial viral infection indicated in 3C2 staining, and appeared within different sections of the same gut in two out of the three guts analyzed. Since the two phenotypes appeared within the same gut tube on multiple occasions, it is possible that the genetic processes occurring within different sections of the small intestine vary, and therefore, the subsections of the small intestine respond uniquely to *Hoxd13* misexpression. It is also possible that the area of viral infection could impact the phenotype of the *cHoxd13*-infected small intestine. There was a slight concentration of viral infection within the mucosa of the gut section expressing the flat, blunted villi phenotype. Potentially, the presence of increased viral infection in the mucosa could cause different patterning pathways to be affected in comparison to when the virus is evenly distributed, as in the gut section expressing the bulbous villi phenotype.

The epithelium in the virally infected ceca section of the gut tube appeared inconsistent and abnormal in comparison to the wildtype gut. The buckling pattern of the ceca epithelium in the *cHoxd13*-infected gut was not continuous, with the bumps varying

in size and shape. This result suggests that *cHoxd13* affects the signaling required for normal ceca epithelium formation to occur at this stage of the developing chick. Alternatively, *cHoxd13* could be required only for normal submucosa formation to occur. If this is the case, the irregular epithelium observed in the ceca of the virally-infected embryo could be solely the result of the abnormal development of the adjacent submucosa. The abnormally thin submucosal layer may have impacted the quality and quantity of the buckling in the epithelium.

Hoxd13 **misexpression leads to phenotypes in the submucosa**

In this experiment, significant divergence from the wildtype phenotype was observed within the submucosal layer of *cHoxd13* virus-infected chick embryos in the gizzard, small intestine and ceca. Discussion of abnormal phenotypes in the submucosa is absent in previous work (Roberts et al., 1998). Abnormalities within the submucosa of injected guts may be indicative of the role of *Hoxd13* in differentiating germ layers in the developing embryo. It may also explain the abnormal phenotypes observed in the endoderm.

In the *cHoxd13*-infected gizzard, large, vacuole-like structures were observed in the submucosa. The function of these vacuoles is yet to be determined. There was disorganization in the germ layers in the infected guts, as it was difficult to determine the beginning and end of each layer. The epithelium was especially thin in the infected gizzard, with the submucosa occupying much of the space normally reserved for the epithelium.

The large quantity of the blood cells in the bulbous villi observed in the small intestines of *cHoxd13*-injected guts indicates interference in submucosa development. In this phenotype, the submucosal layer was not discernable from the epithelial layer. It is possible that viral infection caused the epithelial layer to be thinner, and consequently, the red blood vessels observed in the villi are present because the submucosal layer has been pushed up to occupy the space within the villi. This implies that the abnormally shaped villi and epithelium of the gizzard, small intestine and ceca were the direct result of abnormal submucosa underneath a thinned, but otherwise morphologically normal, epithelial layer.

In the ceca of the virally-infected guts, the submucosa appeared abnormally thin in contrast to the large, disorganized submucosal layer observed in the gizzard and small intestine. This variation in phenotype between gut tube sections could be the result of distinct signaling factors present in each area. *Hoxd13* could potentially be interacting with pathways within the infected tissue and inducing paracrine effects which cause morphological changes within the tissue layers.

The phenotypes related to the submucosa in the gizzard, small intestine and ceca in virally infected guts correspond with varying degrees of viral infection in the submucosa. Strong viral infection in the submucosa within the flat, blunted villi phenotype in the small intestine suggests there is a paracrine effect of the *Hoxd13* virus on the developing germ layers of this area of the chick embryo gut tube. The presence of paracrine signaling effects due to *Hoxd13* misexpression is consistent with the previous findings which quantified and identified expression patterns of signaling factors *Wnt5a* and *lumican*. Both *Wnt5a* and *lumican* are exclusive to the mesoderm of the midgut, and

were present in normal quantity and location within the midgut despite the ectopic expression of *cHoxd13* (Roberts et al., 1998). Normal expression of *Wnt5a* and *lumican* within the same layer of viral expression may suggest that the effects of the virus are seen in nearby layers instead of within the layer of infection. Further, since the normal midgut and foregut inductive factors remain present in the small intestine regardless of ectopic *Hoxd13*-expresion, the resulting phenotype may reflect mixed character of midgut and hindgut instead of pure transformation to hindgut character.

Despite this support of paracrine effects in the small intestine, it is important to note that abnormal epithelium phenotypes in the small intestine, gizzard and ceca were observed concurrently with viral expression within the submucosa and epithelium. Therefore, neither the possibility of *Hoxd13* interference in inter-layer signaling pathways nor interference in intra-layer processes can be dismissed. In many cases within the small intestine and ceca, there was even distribution of viral infection across tissue layers. Consequently, it cannot be determined whether the phenotypes observed within the submucosa and epithelium of these guts were the result of viral infection within the same layer, or viral infection in the adjacent layer. It remains a possibility that *Hoxd13* is involved in regulating the factors involved in mesenchymal-epithelial interaction (Roberts et al., 1998).

Future Work

Analysis of morphological phenotypes in the gizzard, small intestine and ceca of *cHoxd13*-virus-infected guts supports that the role of *Hoxd13* in the developing chick embryo is to determine hindgut morphology. However, further examination of the

resulting phenotypes should be conducted to gain a deeper understanding of the gene function and the specific morphologies controlled by the gene.

Future research should include a repetition of the experiment using a control virus consisting of the same RCAS vector, but lacking the *Hoxd13* sequence. This experiment would identify any phenotypes resulting from the presence of the viral vector and not from the misexpression of *Hoxd13*, and could therefore potentially clarify the function of our gene of interest.

Additional future work will include counting acidic mucin goblet cells within the small intestine of the infected guts, which is critical in understanding the hindgut physiology induced by the *cHoxd13* virus. Acid mucin goblet cells are specific to the hindgut, and are therefore a marker of hindgut physiology. The presence of acidic mucin goblet cells in the small intestine of the virus-infected embryo would further support that misexpression of *Hoxd13* transforms the small intestine to hindgut morphology.

A knock-down experiment of *Hoxd13* in the chick model would also provide insight into the function of the gene. Comparison of phenotypes resulting from a loss-of-function experiment and those of the gain-of-function experiment conducted in this study would help identify the components of the gut tube controlled by *Hoxd13*. To perform this knock-down experiment, we would construct a virus expressing small interfering RNA specific to *Hoxd13*. This virus would silence the expression of *Hoxd13* by degrading all mRNA transcripts of the gene.

Evolutionary conservation is another aspect of *Hoxd13* which will be interesting to further understand. As described earlier, I created an RCAS viral vector containing the skate ortholog, *LeHoxd13*. Follow-up work in our lab will including repeating the

experiment described in this paper using the RCAS vector containing *LeHoxd13*. If the skate virus produces the same phenotypes as the *cHoxd13* virus, it can be deduced that the function of *Hoxd13* has been evolutionarily conserved since the evolution of the cartilaginous fish. Knowing when this gene evolved will provide insight into the evolution of the *Hox* gene family and the vertebrate digestive system. This knowledge could potentially serve in understanding and treating diseases linked to *Hox* genes and digestive tract development and function.

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