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Analysis of Differential Gene Expression Patterns During Spiral Intestine Development in *Leucoraja erinacea*

By

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of the requirements for

Honors in the Department of Biological Sciences

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Abstract

Advisor: Nicole Theodosiou

The vertebrate gut is a specialized structure responsible for the intake and digestion of food, absorption of nutrients, and expulsion of waste products. The organs of the digestive tract have been conserved over time, but modifications to the size and shape of individual structures exist within the different vertebrate lineages. The skate's spiral intestine has evolved to maximize nutrient absorption within a compact structure to create space in the body cavity for the organs needed for buoyancy. Studying the unique intestinal morphology of *Leucoraja erinacea,* or the little skate, provides an opportunity to understand the role of differential gene expression in development. The vertebrate digestive system develops from the embryonic gut tube through the expression of specific genes that direct the development of different tissue types and organs at specified locations. The genes *Shh, Wnt5a, Sox2, Hoxa13, Hoxd12,* and *Cdx2* are necessary to pattern the intestines in other animals including mice, chick and human. We have cloned these genes from the skate and are characterizing their expression by RNA whole mount in situ hybridization in the developing spiral intestine. The results will allow us to generate a profile of the conserved genes involved in intestinal differentiation. By comparing gene expression profiles among different vertebrates, we can link information at the molecular level with the structural and functional adaptations that have evolved in the digestive tracts across lineages. Therefore, the study of differential gene expression in embryonic development can provide an opportunity to understand events of vertebrate evolution dating back 400 million years

Introduction

The vertebrate gut is a specialized structure responsible for the intake and digestion of food, absorption of nutrients, and expulsion of waste products. The organs of the digestive tract are conserved over time, but modifications to the size and shape of individual organs exist within the different vertebrate lineages. The mature digestive tract is a continuous pathway through the body cavity from the mouth to the anus. The two portals of entry and exit are connected through a series of hollow organs responsible for the breakdown, movement, and absorption of nutrients. Each organ has a specific form and function based on the organ's location along the digestive tract and therefore its function in digestion. The established order of organs along the gastrointestinal tract requires the proper functioning of each preceding segment in order to successfully digest material and extract nutrients. Therefore, examining the digestive tract as a whole is necessary to understand the origin and function of each individual structure.

Variations in the structure and size of the digestive tract have evolved to suit different aspects of an organism such as its environment, nutritional niche, and body structure. For example, the fish's aquatic environment requires that it must pass large volumes of water through the oral cavity and gills in order to respire, therefore limiting the potential routes by which small food particles can be ingested (Stevens and Hume, 2004). A nutritional niche is a combination of the organism's energy / nutritional requirements for survival and the mechanisms the organism uses to obtain these nutrients (Stevens and Hume, 2004). The specific diet of an organism often correlates to features of its digestive tract. In comparing the length of different fish digestive tracts, the herbivorous species tended to have the longest intestinal tracts in order to deal with indigestible mud or plant material that gets ingested (Stevens and Hume, 2004). In contrast to fishes, the avian body structure is specialized for flight, which restricts the

distribution and weight of the gut contents. The avian esophagus is long and wide with a specialized crop structure used for food storage (Stevens and Hume, 2004). As vertebrate species have both colonized and relocated to inhabit different ecosystems throughout history, the digestive tract organs have evolved to take on a specific form and function that meets the demands of the organism's lifestyle. Through the study of structural and functional differences that have evolved in the digestive tracts across different lineages, we are able to create an understanding of vertebrate evolution dating back 400 million years.

Evolution of the vertebrate digestive tract can be linked to developmental steps at the molecular level. The vertebrate gut is initially a straight and uniform tube that acquires shape through differential gene expression. Therefore, the gut provides a complex model to study gene patterning during embryonic development. Specific genes are expressed at different locations based on their role in developing that section of the gut. Comparing and contrasting the gene expression patterns across species synthesizes a guide to observe how a certain gene's characteristic expression pattern results in that organism's unique organ morphologies. The research also supplements an understanding of the role that conserved genes have on development throughout vertebrate gut evolution.

The anterior-posterior axis begins at the rostral end of the gut tube and extends to the caudal end. Gut tube formation begins with two invaginations occurring at the anterior end (anterior intestinal portal, AIP) and the posterior end (Caudal Intestinal Portal, CIP) of the tube, respectively (Sanderson and Walker, 1999). The two invaginations form each end of the prospective digestive tract. Subsequent growth of endoderm at the two invaginations toward one another in combination with embryonic turning events allow the two ends to fuse, forming one tube composed of endoderm (Wells and Douglas, 1999). A layer of splanchnic mesoderm

overlies and encircles the endoderm tube, establishing proximity for the two layers to interact. Coordinated signaling along the A-P axis of the primitive gut tube gives rise to organs defined by the expression of differential genes in the endoderm and mesoderm for that given location. The organs of the foregut (esophagus, stomach, and lungs), the midgut (small intestine) and the hindgut (colon and rectum) all develop from the embryonic gut tube (Smith et al, 2000). Specifically, it is the epithelial-mesenchymal interaction along the A-P axis that allows for the histologically uniform gut tube to develop into the three functional regions of the gastrointestinal tract (Roberts et al., 1998).

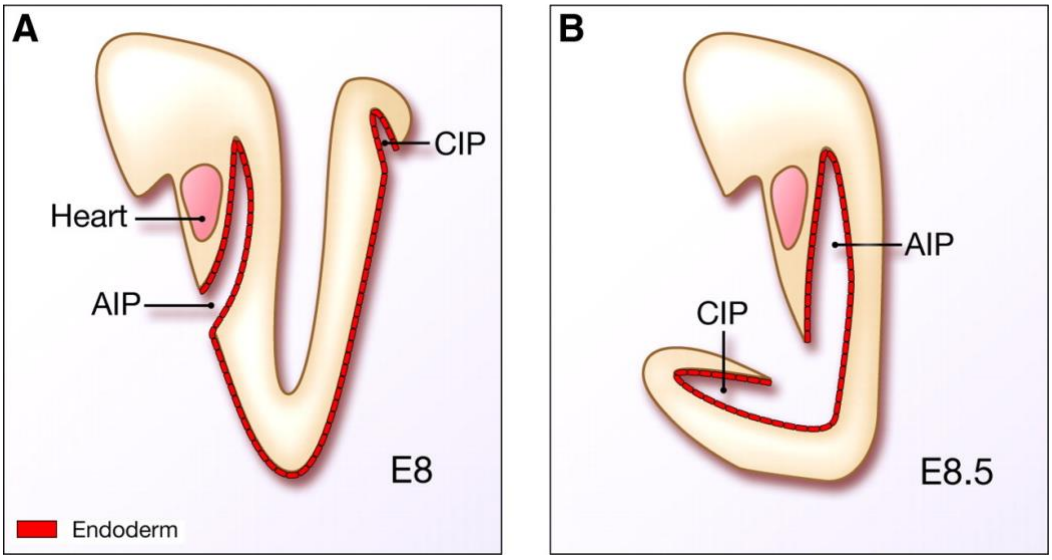

Figure 1. A) The first step in the formation of the embryonic gut tube. Invaginations of the definitive endoderm at the anterior (anterior intestinal portal, AIP) and posterior end (caudal intestinal portal, CIP) initiate internalization of the gut. B) Embryonic turning events orient the two invaginations such that they can fuse to form a closed tube lined with endoderm (Image: van de Brink, 2007).

The exchange of signals between the endoderm and mesoderm during vertebrate embryonic development is critical to gut tube formation. An array of signaling molecules work together to provide instruction for how the digestive tract develops. One gene that plays a large part in development is *Sonic hedgehog (Shh)*, which encodes a protein that acts as an inductive

signal expressed in the endoderm of the developing gut. A homolog of the *Drosophila* gene *Hedgehog (hh), Shh* is thought to be conserved across vertebrates. *Shh* expression is first detected in the endodermal regions of the AIP and CIP in the early stages of gut formation (Roberts et al., 1995). As previously noted that the induction of the AIP and CIP initiates development of the digestive tract, *Shh* is therefore hypothesized to serve a critical role in the epithelial-mesenchymal interactions that govern development by inducing *Bmp-4* expression in the splanchnic mesoderm that will give rise to the smooth muscle of the gut (Sanderson and Walker, 1999). Following the initial expression of *Shh* at the invagination sites, *Shh* expression expands and is maintained in the gut endoderm as morphogenesis of the gut tube proceeds (de Santa Barbara et al., 2002).

Endoderm-secreted *Shh* acts through the *Patched (Ptc)* receptor expressed in the mesoderm to induce mesodermal expression of *Bmp-4* (de Santa Barbara et al., 2002). The interaction between *Shh* and *Bmp-4* was predicted to function in the epithelial-mesenchymal signaling that results in early hindgut formation. *Shh* in the chick hindgut is sufficient to induce ectopic expression of its target molecule *Bmp-4 Bmp-4* in the undifferentiated mesoderm adjacent to where *Shh* exists in the endoderm(Roberts et al., 1995). The inductive signaling potential for *Shh* in the developing gut is further confirmed by observing that virally mediated expression of *Shh* is able to induce *Bmp-4* in the visceral mesoderm (Roberts et al, 1995).

The study of *Shh* signaling during the development of the vertebrate gut is built upon by studies conducted with mice. The comparison of wild type and mutant mice for *Shh* revealed obvious phenotypic abnormalities spanning the majority of the gastrointestinal tract. Mice mutant for *Shh* displayed a marked overgrowth of stomach epithelium, malrotation of the gut, and impairments to the smooth muscle located along the small intestine (Ramalho-Santos et al.,

2000). The results indicate that *Sonic Hedgehog* functioning is therefore essential for successful organogenesis in mice. Due to the fact that *hedgehog* genes are expressed in the gut endoderm in all vertebrates, the implication that *Shh* expression could be linked to gastrointestinal malformations provides motivation for understanding how the gene functions in developmental pathways. Investigation of *Shh* in producing an abnormal gut phenotype offers a research avenue for the link between signaling pathway mutations and common gastrointestinal irregularities observed across species.

The *Hox* genes are an important group of potential downstream targets for *Shh* during gut regionalization along the A-P axis. The *Hox* genes comprise a family of transcription factors that play a large role in development. Many of the genes function similarly in vertebrate development across species, indicating that *Hox* gene function has been conserved throughout vertebrate evolution. Coordinated induction of *Hox* genes along the body axes help direct body plan design (Illig, Fritsch and Schwarzer, 2012). *Hoxd12* is an example of a gene thought to be conserved in body plan development as it is proposed to function in vertebrate limb development (Cho et al., 2008). Additionally, in the context of gastrointestinal tract development, different *Hox* genes are located in the mesoderm and endoderm at distinct regions along the gut tube to define organ identity. Studies on mouse and chick embryogenesis have reported *Hoxd12* expression in the anal sphincter and terminal gut mesoderm, suggesting that *Hoxd12* may have a varying function in different organisms (Mark et al., 1997)

The various *Hox* genes associated with gut tube patterning have been suggested to play a part in establishing demarcations between the major anterior-posterior axis regions (Roberts, 2000). Another *Hox* gene identified for its role in gut development is *Hoxa13*. Specifically, *Hoxa13* is expressed in both the chick cloaca endoderm and mesoderm, with a more expansive

expression range within the endoderm that gives rise to the hindgut in chick (de-Santa-Barbara and Roberts, 2003).

The conserved nature of *Hox* genes leads us to believe that *Hoxa13* will function similarly in developing the hindgut in other vertebrates. Research on *Hox* gene expression throughout human embryonic development reported that expression levels for genes within the different *Hox* clusters fluctuated based on the developmental stage under observation (Illig, Fritsch and Schwarzer, 2012). Therefore, during our study of *Hox* genes on gut tube development it will be important to repeat the experiment with embryos at different stages in development. Analyzing the differences in function within the highly conserved gene family can reveal that although specific genes are crucial to gastrointestinal tract development, functional variation exists to give rise to each organism's distinct organ morphology.

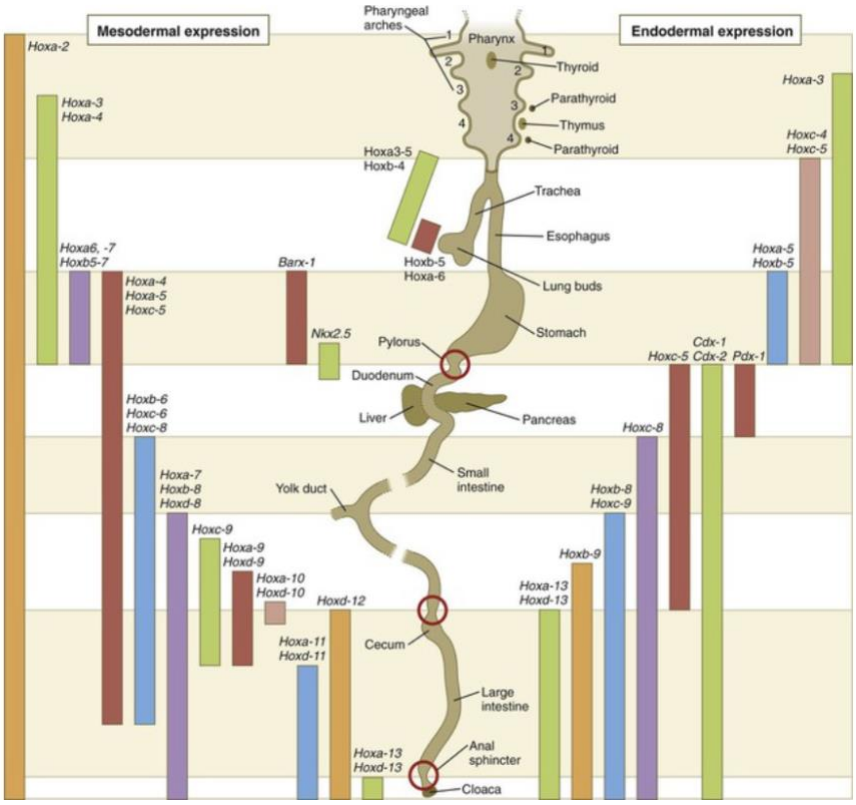

Figure 2. *Hox* gene expression location in the mesoderm and endoderm along the developing gastrointestinal tract (image from Clinical Gate, 2015).

Alteration of gene expression patterns during embryogenesis and subsequent phenotypic analysis can provide information about a gene's specific function. Another gene identified for its role in gut development is *Wnt5a.* The importance of proper *Wnt5a* expression is illustrated through a study conducted on mice mutant for *Wnt5a* in which the small intestine was dramatically shortened and the lumen bifurcated as opposed to a single tube (Cervantes, 2009). The mice displayed a marked defect in posterior elongation, which was responsible for the shortened small intestine. Based on this result, *Wnt5a* has been proposed to facilitate proper cell proliferation during development. Further, in the absence of *Wnt5a,* cell proliferation was reduced by 36% and 16.2% in the epithelial and mesenchymal regions, respectively. The finding that mutant mice displayed irregular phenotypes affirms that *Wnt5a* is necessary for gut tube development (Cervantes et al., 2009). The exact mechanism of molecular *Wnt5a* regulation throughout development is continually being investigated.

As the gut regionalizes along its AP axis, differential gene expression gives rise to demarcations that dictate the boundaries between regions. Examining which genes act as molecular markers provides context for the gene's expression range along the gut tube. For example, the hindgut of most fish is not readily distinguished from the midgut by the diameter or changes in epithelium, therefore a molecular marker is useful for highlighting this demarcation (Stevens and Hume, 2004). Along with its role in small intestine elongation, *Wnt5a* can also be utilized as a molecular signal that specifies the boundary between the small and large intestine in some lineages (Roberts et al., 1998). Wnt signaling also works in activating transcription factors like *Cdx2* that differentiate the gut tube (Sherwood et al, 2011).

The vertebrate *Cdx* genes code for homeodomain transcription factors involved in intestinal development and homeostasis. *Cdx2* is important for both the development and maintenance of the small intestine (Costkun et al., 2011). *Cdx2* acts as an early molecular marker of intestinal fate and is responsible for differentiating between the midgut and hindgut; a high concentration of *Cdx2* leads to patterning of the large intestine while a lesser concentration promotes formation of the small intestine (Gilbert, 2016). The crucial role of *Cdx2* in development is evident through experimentation on mice embryos with inactivated *Cdx2* alleles. While homozygous knock-out mice were embryonic lethal due to an implantation failure, *Cdx2* heterozygotes were viable, but the colon and small intestine developed lesions and a gastric-like epithelium (Costkun et al, 2011).

Cdx2 is also necessary for homeostasis and continued maintenance of the intestinal mucosa. Specifically, *Cdx2* expression has been linked to different gastric cancers for its potential role as a tumor suppressor gene. In a sample of surgically dissected gastric tissue collected from human patients, one study found that *Cdx2* expression was closely associated with gastric epithelial dysplasia (Seno et al, 2002). Patients with the *Cdx2*-positive phenotypes had a longer survival period compared to those without it, suggesting a potential role for *Cdx2* as a tumor suppressor. Additionally, overexpression of *Cdx2* was found to inhibit the growth of colon cancer cells in vivo (Seno et al, 2002). This finding necessitates further investigation on the role of proper *Cdx2* in development as colon cancer ranks among the top 3 most common types of cancer (World Cancer Research Fund). Due to the conserved nature of gene expression across vertebrates, research on *Cdx2* can offer an opportunity for work with model organisms to learn more about the cellular abnormalities of different diseases.

Whereas *Cdx2* is expressed in the posterior part of the primitive gut, *Sox2* is a transcription factor expressed in the anterior portion of the primitive gut. The close expression proximity and reciprocal expression patterns of *Cdx2* and *Sox2* along the digestive tract pose the question of whether the two transcription factors influence one another in specifying endoderm fate. Induction of ectopic *Sox2* expression in prospective mouse intestinal cells resulted in a swollen, fluid-filled intestine with abnormal villi (Raghoebir et al., 2012) Further, the ectopic *Sox2* expression resulted in an anteriorization of intestinal epithelium as a consequence of newly expressed esophageal and stomach specific molecular markers within the intestine. Induction of *Sox2* in the intestinal epithelium did not affect the expression levels of *Cdx2,* but did affect *Cdx2* functionality. Analysis of the binding efficiency of *Cdx2* to the promoter of two of the transcription factor's known target genes, *Cdh17* and *Hnf4a,* revealed that in the presence of ectopically induced *Sox2* expression, there is a significant decrease in the activation of the *Cdx2* target genes (Raghoebir et al., 2021). *Sox2* therefore exerts a dominant influence over *Cdx2* such that *Sox2* causes unprecedented activation of foregut transcriptional machinery, resulting in anteriorization of the gut.

Over or under expression of a gene in its characteristic location can have adverse effects on development. The relationship between *Sox2* and *Cdx2* illustrates the importance of regulating expression levels so appropriate specification of the gut tube can ensue. The ability of *Sox2* to interfere with the function of *Cdx2* as a transcriptional regulator demonstrates how molecular signals can interact with one another during embryonic development. The location that specific genes are expressed along the primitive gut tube has a powerful effect on the fate of that section during embryogenesis. Therefore, mis-expression can have detrimental effects on developing a functional digestive tract.

The flow of digestive material from the beginning of the digestive tract to the end makes it such that the action of one organ is dependent on the proper functioning of the organ that precedes it. Therefore, establishment of the first segment of the digestive tract, the foregut (esophagus, stomach and lungs), is necessary to ensure the entire digestive system can function. The location of *Sox2* expression in the anterior portion of the primitive gut tube makes the gene a key component in the interaction between the mesoderm and endoderm that is responsible for foregut specification. Patterning along the length of the tube establishes the demarcations of different molecules and transcription factors that will lead to development of structures such as the trachea and esophagus. Expression of *Sox2* and *NKX2.1* in the correct location along the gut tube ensures separation of the esophagus and trachea, and subsequently the establishment of the initial structures of the respiratory and digestive system (Zhang and Cui, 2014). *Sox2* is expressed in the dorsal epithelial cells of the unspecified foregut tube, while *NKX2.1* the ventral epithelium. Down regulating either *Sox2* or *NKX2.1* results in the expansion of the expression pattern characterized by the other (Zhang and Cui, 2014). The relationship between *Sox2* and *NKX2.1* highlights the influence of gene interaction on the formation of both an intact respiratory and digestive system.

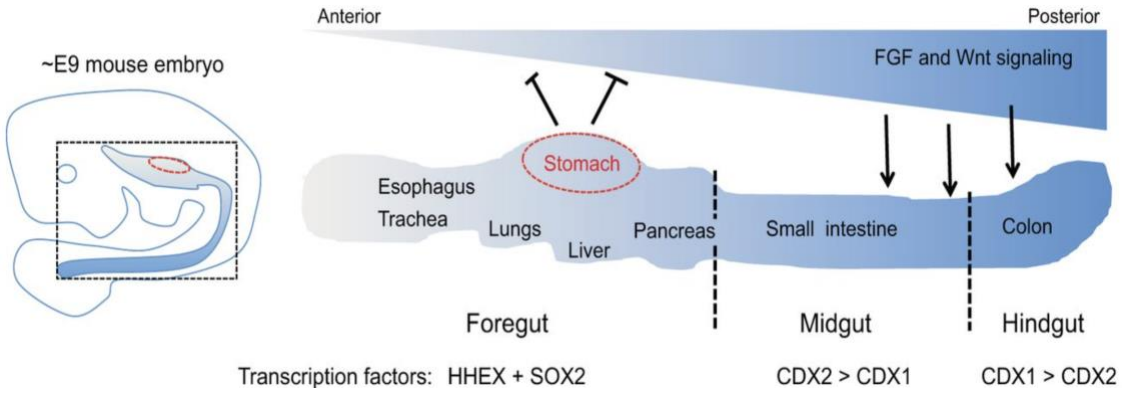

Figure 3. *Sox2* and *Cdx2* expression patterns in the mouse embryo. Gene interaction and expression levels determine where the demarcations of the foregut, midgut and hindgut fall along the embryonic gut tube (Kim and Shivdasani, 2016).

The digestive tract of cartilaginous fish (belonging to the class Condrichthyes and subclass Elasmobranchs including rays, sharks and skates) displays a unique intestinal morphology. The intestine has evolved as a way for the fish to adapt to an aquatic lifestyle while maintaining sufficient nutrition. Elasmobranchs need to remain buoyant in water and achieve this via two large liver lobes that take up the majority of their body cavity. Therefore, the digestive tract has evolved in order to minimize the space it occupies within the body by forming a spiral intestine, resembling that of a corkscrew. Compared to teleost fish, the elasmobranchs have a significantly smaller than expected intestinal length (Shadwick et al., 2016). Relative length of intestine to body cavity ratios reveal an 8:1 and a 3:1 difference between herbivorous teleost fish to elasmobranchs and carnivorous teleost fish to elasmobranchs, respectively (Shadwick et al., 2016). The spiral structure allows for optimal absorption of nutrients without taking up vital space needed for other functions. Additionally, the spiral valve is proposed to function in slowing down the transit time of digested material (Shadwick et al., 2016). A slower transit time of material along the gastrointestinal tract presents an opportunity for increased food processing to ensure sufficient absorption within a smaller than expected intestine.

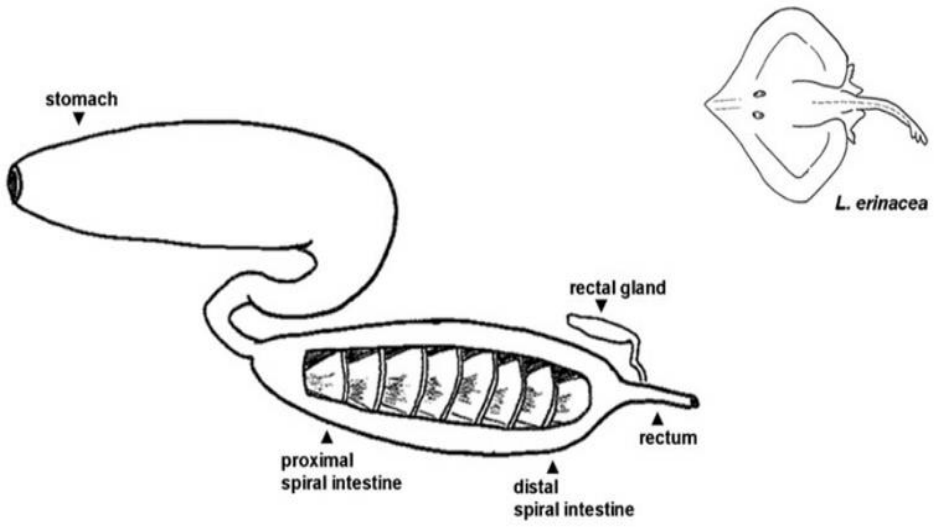

Figure 4. Visual representation of the unique intestine morphology in the little skate, *Leucoraja erinacea*. The shape of the spiral intestine is analogous to the shape of a corkscrew **(**Theodosiou et al., 2012).

Studying the intestine of *Leucoraja erinacea,* or the little skate, provides an opportunity to examine the distinct gene patterning that resulted in the adaptation of the spiral valve. Furthermore, comparing the expression patterns of the genes involved in development of the unique skate digestive tract to the known expression pattern of other vertebrates can reveal information about vertebrate evolution. The genes *Wnt5a, Cdx2, Sox2, Hoxa13* and *Shh* have been cloned in the lab from the skate, which allows for the generation of RNA probes for each gene. By using a labeled RNA strand complementary to the DNA coding sequence of the gene of interest, we are able to visualize where in the body different genes are expressed. Whole mount RNA *in situ* hybridization can be used with each specific gene probe to determine where a gene is active. Further sectioning and staining of the embryo may show what germ layer the gene is active in during progressive stages of development. Creating a holistic picture of where and when genes are expressed creates an understanding of how the gut tube is patterned in the little

skate. Comparing gene activity patterns then determines which genes have been conserved across lineages, and which genes have been modified over the course of vertebrate evolution.

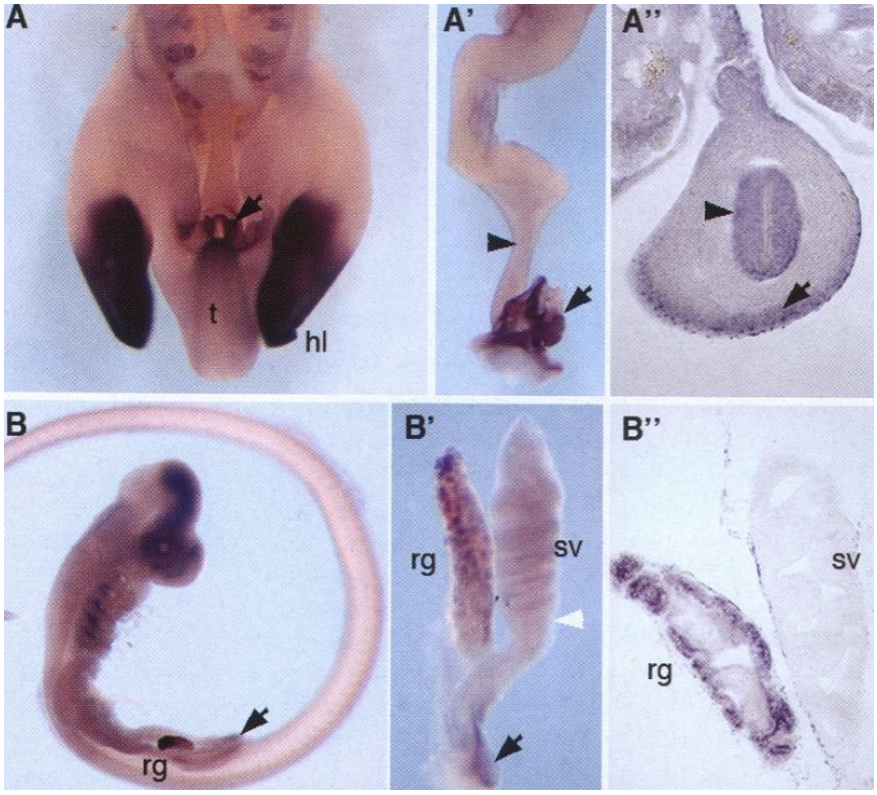

Figure 5. Example of whole mount RNA *in situ* of *Hoxa13* in the little skate. The dark purple staining is indicative of gene expression in that region. The expected staining pattern for *Hoxa13* is evident in the hindgut (Theodosiou et al., 2007).

Materials and Methods

Gene cloning and sequence confirmation

The genes *Shh, Wnt5a, Cdx2* and *Sox2* were previously cloned in Professor Theodosiou's lab and isolated from *L. erinacea* by Sam Frye, Loren Collado, and Jake Alexander. Following PCR amplification from a single-stranded cDNA template, the DNA PCR-amplified fragments were ligated into the pGEM-T easy vector (Figure 6). Bacterial vectors with cloned genes were transformed into *E. Coli* competent cells, and . 10%, 30% and 60% of the transformation reaction product was expelled onto individual LB-Agar/Carbenicillin/X-gal plates. Plates were

incubated in a 37°C oven overnight for colony growth. Following incubation, isolated colonies were transferred from the plates into snapcap tubes containing 2.5 mL of LB treated with carbenicillin. Tubes were incubated in a 37°C oven overnight, shaking. The following day, cells were isolated and mini-prepped using the protocol provided with the Qiagen mini-prep DNA isolation kit.

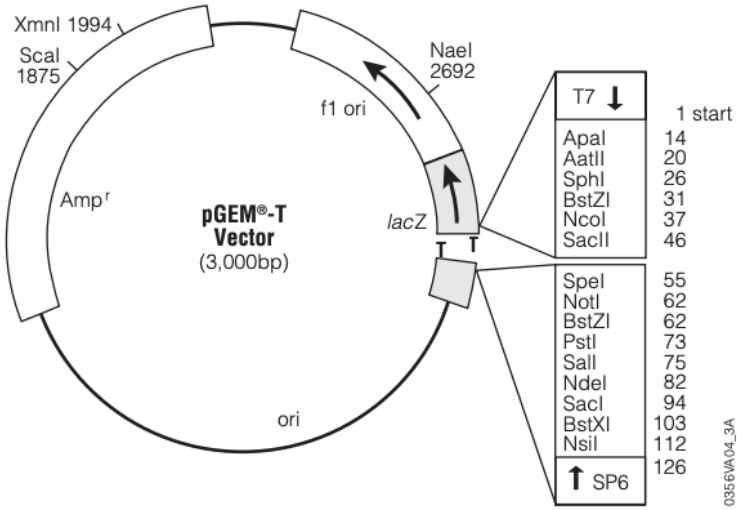

Figure 6. **pGEM®-T Vector** pGEM®-T Vector Map and Sequence Reference Points (pGEM®-T and pGEM®-T Easy Vector Systems, 2018).

To confirm that the isolated genes were cloned into the pGEM vector, we cut each miniprep sample with EcoRI. The restriction enzyme digests consisted of 15 µL miniprep DNA, 3 µL Buffer H, 1 µL enzyme EcoRI, and 11 µL dH2O in microcentrifuge tubes. Tubes were incubated in a 37°C water bath for 1 hour and 30 minutes. 30 µL of digest and 6 µL of EZ-dye were loaded into the lanes of a 0.8% agarose gel along with a 1kb ladder run at 100mV. Imaging of the gels revealed that the vector (roughly 3,000bp or 3kb) and the gene inserts (*Shh, Wnt5a, Sox2*) were cut and separated (Figure 7).

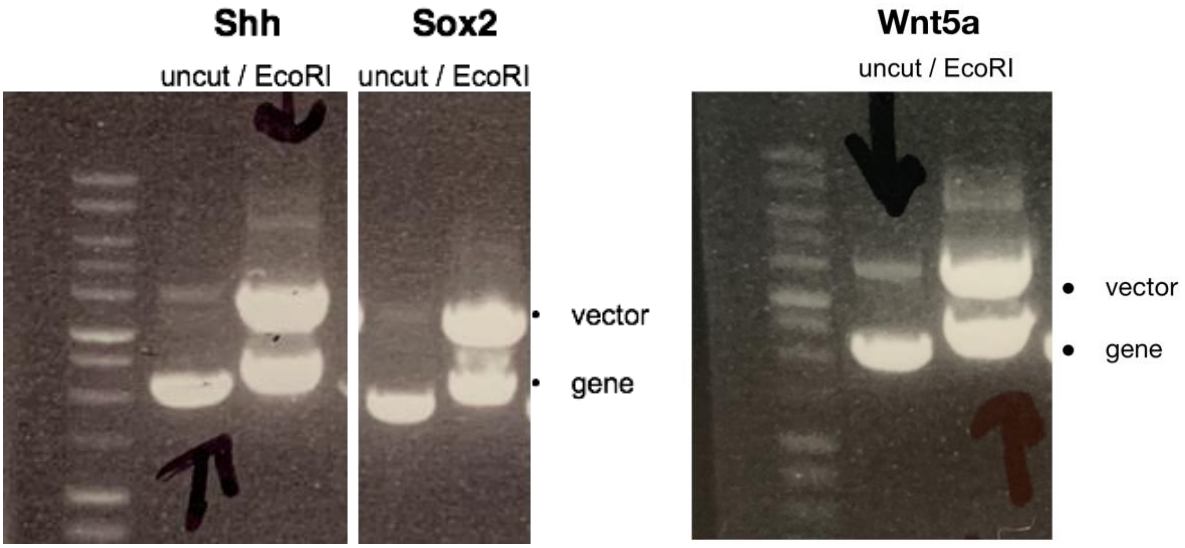

Figure 7. 0.8% agarose gel of mini-prep *Shh*, *Sox2* and *Wnt5a* DNA cloned from skate ("uncut"). Restriction enzyme digests performed with EcoRI restriction enzyme cut out gene insert from the pGEM vector. Vector appears to be roughly around 3kb as expected. The first lane is a 1kb ladder.

Samples were quantified using the NanoDrop machine to assess the purity of the *Shh, Sox2* and *Wnt5a* DNA. Samples for each gene were then prepared and sent to GENEWIZ Biotechnology Company in South Plainfield, New Jersey. The sequence data returned confirmed the identification and orientation of the isolated *Shh* and *Sox2* genes. The genes were oriented in the pGEM vector with *Sox2* 5' at the T7 polymerase site, and *Shh* 5' at the SP6 site. We used information provided on the pGEM-T Easy Vector System guide to identify unique restriction enzyme sites on the 5'-end of the gene inserts, and subsequent RNA polymerase to use for constructing an RNA probe from the DNA (Table 1). The sequence data for the gene we thought was *Wnt5a* aligned instead with the gene transcript for *Wnt5b.* Synthesis of primer sequences for the *Wnt5b* gene are needed in order to proceed with investigation of the role of the gene in skate.

RNA probe synthesis

The 5' end of *Sox2* was oriented in the vector at the T7 polymerase primer site, which prompted us to use NcoI, a unique site on the 5' end of the gene insert, for the restriction enzyme

digest. 15 µL mini-prep *Sox2* DNA, 3 µL buffer H, 1 µL NcoI and 11 µL dH2O were incubated in a 37°C water bath for 1 hour and 30 minutes. The digests were then run on a 0.8% agarose gel to confirm that the DNA was cut (Table 1, Figure 8). Next, a transcription reaction of 8 µL of the enzyme digest, 6.5 µL dH_2O, 2 µL 10X transcription buffer, 2 µL 10X nucleotide mix (DIG), 0.5 µL RNAse inhibitor and 1 µL SP6 RNA polymerase was incubated in a 37°C water bath for 2 hours. We ran 1 µL of the product on a 0.8% agarose gel to confirm that the reaction was complete (Figure 9). The probes were then precipitated by freezing 1 µL DNase, 80 µL dH_2O, 10 µL 4M LiCl and 330 µL 100% EtOH for 30 minutes, then spinning and washing the samples with 70% EtOH, and lastly dissolving the samples in 100 µL dH_2O to be stored at -20°C until use.

The same steps were used to synthesize the *Shh* probe, with a notable exception to the restriction enzyme and RNA polymerase utilized. Based on the GENEWIZ sequencing results, the *Shh* orientation in the vector was 3' → 5', which prompted us to utilize Sal1 as the restriction enzyme and T7 as the RNA polymerase (Table 1). However, when run on an agarose gel, Sal1 did not appear to have successfully cut the DNA and the restriction enzyme digest was redone with Pst1 (Figure 8).

Following the same steps used for synthesis of the *Sox2* probe, we used Not1 and T7 for the restriction enzyme digest and subsequent transcription reaction with the *Cdx2* DNA that we mini-prepped, although the DNA was never sent out for sequencing (Table 1). We ran a transcription reaction with the cloned EST #3837 of *Hoxa13* DNA with RNA polymerase T7 and precipitated / re-suspended the probe in the same manner as the other genes (Tabe 1).

In order to compare the expression profiles of genes involved in intestinal development across vertebrate species, we synthesized probes for whole mount *in situ* in chick. Using the

linearized vectors with gene inserts, we ran transcription reactions for c*Hoxd12* and c*CdxA* both with T7, confirmed completion of the reaction through gel electrophoresis, and precipitated / re-suspended the probes (Figure, 9). All probes were stored at -20°C until ready for use in the whole mount in situ hybridizations.

Table 1. Restriction enzyme and RNA polymerase utilized for digest and transcription reactions, respectively, for *Sox2, Cdx2, Shh* and *Hoxa13* EST #3837 in skate.

Gene	**Restriction Enzyme**	**RNA Polymerase**
Sox2	NcoI	SP6
Cdx2	NotI	T7
Shh	PstI	T7
Hoxa13 EST# 3837	N/A	T7

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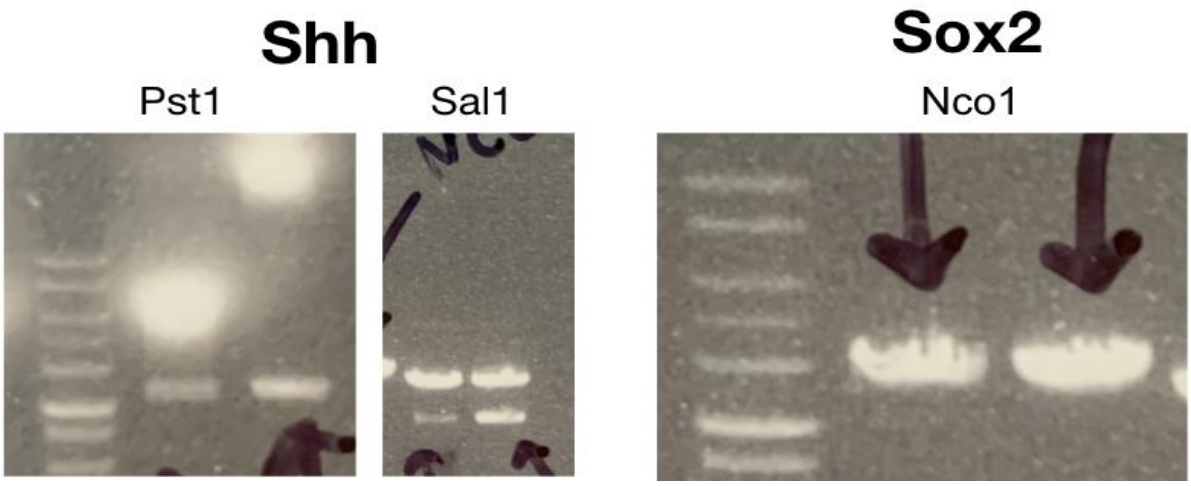

Figure 8. Restriction enzyme digests. 0.8% agarose gel image of Shh restriction enzyme digests. *Shh* cut was initially unsuccessful with restriction enzyme Sal1. *Shh* restriction enzyme digest was repeated with Pst1, which successfully cut and linearized the plasmid. *Sox2* was successfully cut with NcoI. The far left lane in each image is a 1kb ladder.

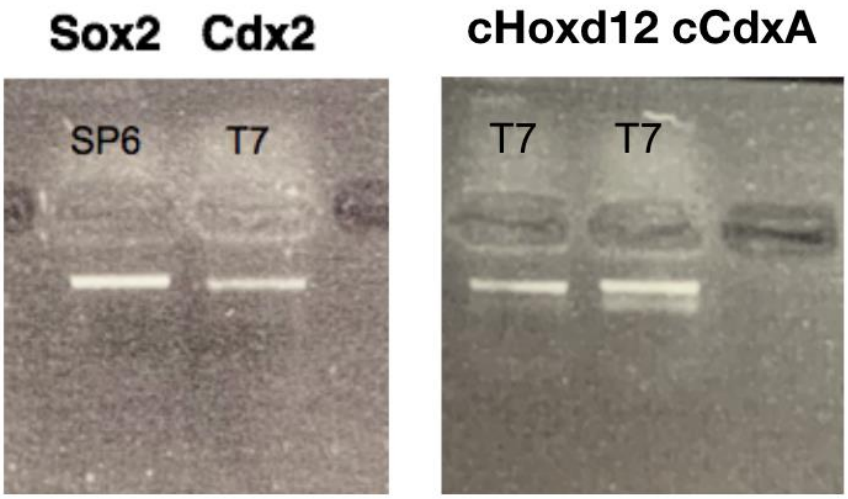

Figure 9. Transcription reaction products. (L) 0.8% agarose gel of the transcription reaction product of *Sox2* and *Cdx2* with RNA polymerase Sp6 and T7, respectively. (R) transcription reaction result for c*Hoxd12* and c*CdxA* both with RNA polymerase T7. Presence of intact band confirms the transcription reaction was complete and we could move on with synthesizing the probes.

Whole Mount in situ RNA Hybridization

Stage 29 chick embryos were used in accordance with the standard Theodosiou lab protocol for whole mount RNA *in situ* hybridization with c*Hoxd12* and c*CdxA* probes. Embryos were treated with 20 ug/mL of proteinase-K for 20 minutes to increase embryo permeability for the probes. Embryos were analyzed and imaged under a microscope following the successful visualization of c*Hoxd12* and c*CdxA* (Figure 10).

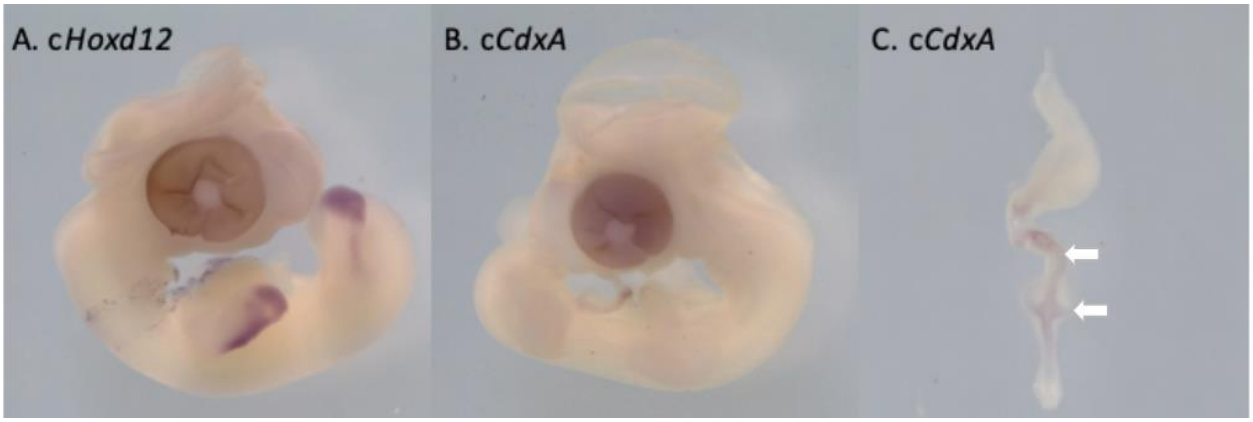

Figure 10. Whole mount *in situ* hybridization images from stage 27 chick embryos. A) *Hoxd12* expression detected in the fore and hindlimb buds. *Hox* genes are expressed in the endoderm and mesoderm of the developing embryo.

B) *CdxA* expression visible in the endoderm of the developing gut tube. C) Dissection of the developing gastrointestinal tract illustrates that *CdxA* is expressed in the intestinal endoderm in the developing chick.

Following the successful whole mount *in situ* trial in the chick, we repeated the experiment in skate. Stage 25 - 27 skate embryos from Marine Biological Laboratory at Woods Hole, MA were used for whole mount RNA *in situ* hybridization to visualize the expression patterns of *Sox2, Shh, Cdx2,* and *Hoxa13* (Theodosiou et al., 2007). The initial whole mount *in situ* experiment with *Sox2, Shh* and *Cdx2* in skate did not produce any definitive staining results (Figure 11).

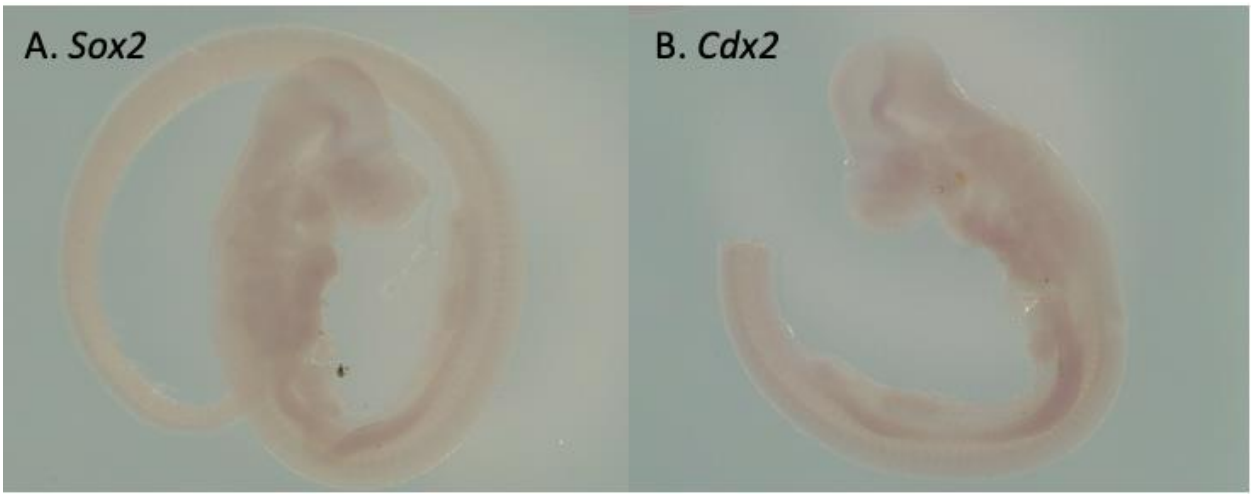

Figure 11. Whole mount RNA *in situ* hybridization results in stage 25 skate embryos. Proteinase-K treatment was 10 ug/mL for 15 minutes. A) No definitive staining pattern evident for *Sox2.* B) No definitive staining pattern evident for *Cdx2.*

The skate embryos used for the *Hoxa13 in situ* were treated with 30 µg/mL of proteinase-K for 15 minutes. Microscopic analysis and imaging of the skates revealed staining as expected (Figure 12). Due to anticipation of staining in the endoderm, it is likely that a stronger concentration and longer duration of proteinase-K treatment was necessary for the probe to penetrate the embryo efficiently. The experiment was re-done with *Sox2* and *Cdx2* in skate, but we were unable to produce definitive expression patterns.

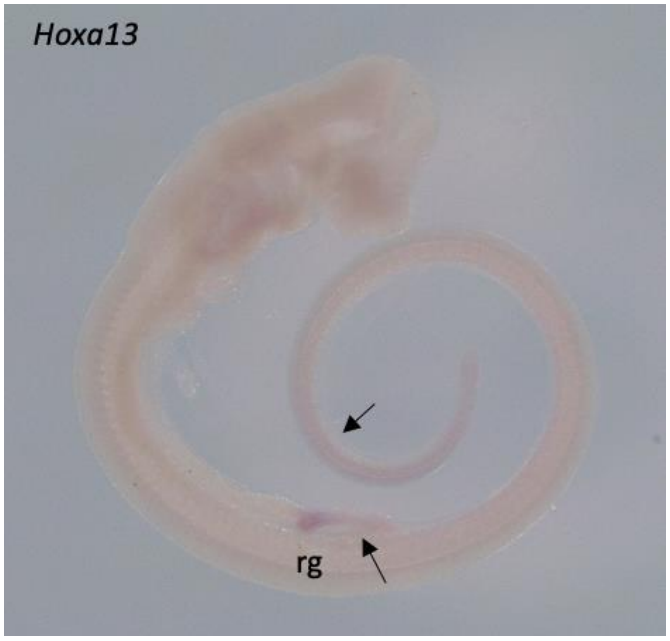

Figure 12. *Hoxa13* whole mount RNA *in situ* hybridization expression in a stage 25 skate embryo. *Hoxa13* expression detected in the rectal gland (rg), cloaca (bottom arrow) and tip of tail as expected. Proteinase-K concentration increased to 30 ug/mL for 15 minutes

Results

The research aims of this project sought to identify previously determined genes conserved in gut tube patterning (*Shh, Wnt-5a, Cdx2, Sox2)*, isolate and clone the genes from the little skate, and synthesize RNA probes for use in whole mount RNA *in situ* to visualize and compare gene expression patterns among vertebrate.

Sequence alignments

Miniprep DNA for *Sox2*, *Shh* and *Wnt5a* were sent out for sequencing. The returned data provided identification sequences for *Sox2* and *Shh* in skate, but revealed that cloning of *Wnt5a* was unsuccessful. An open reading frame finder was used to identify five reading frames for *Sox2*, of which ORF2 had the highest percent similarity to the *Sox2* sequence across vertebrate species, with 88% similarity to the mouse sequence, 84% to chick and 71% to frog. The ORF sequences for *Sox2* in mouse, chick and frog were obtained from https://blast.ncbi.nlm.nih.gov/

and loaded into a multiple sequence alignment tool to illustrate the similarities and differences between the *Sox2* sequence from our sample, mouse, chick and frog (Figure 11). The extent of overlap and therefore similarity of gene sequences emphasizes the conservation of genes involved in vertebrate gastrointestinal development.

While the BLAST (basic local alignment search tool) analysis of the *Shh* data revealed similar conservation patterns among vertebrates, our sample sequence aligned close with the genomic sequence for *Shh* in the little skate, *Leucoraja erinacea*. Alignment of the sample *Shh* sequence and the *Shh* sequence provided for *L. erinacea* had a 97% identity and positive percentage rate, indicative of the residues located in identical positions and conservatively substituted residues, respectively (Figure 12). The similarity of the alignment further confirms that the *Shh* gene was successfully cloned and isolated from the little skate. Sequencing the DNA samples was an important step for generating RNA probes. The sequence data both confirmed the identity of our DNA samples and provided instruction for the restriction enzymes and RNA polymerases needed for the subsequent reactions. Additionally, the sequence data was then compared with other known vertebrate gene sequences through sequence alignment tools. Sequence alignments are useful to our study because they identify the extent of similarity among protein sequences, which can indicate evolutionary relationships among species.

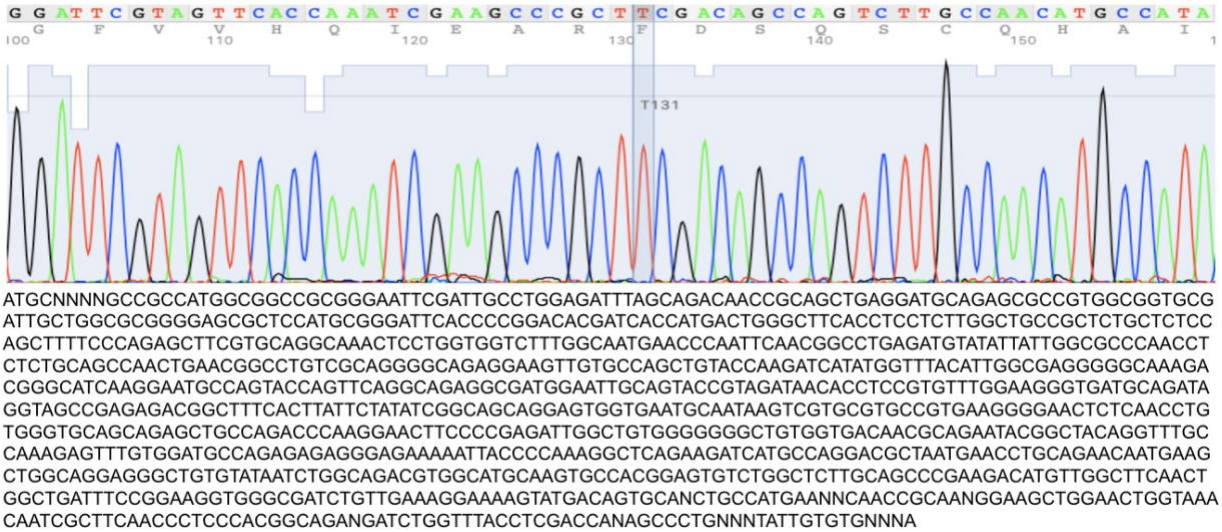

Figure 13. *Wnt5b* Identification. Example segment of the information returned from GENEWIZ and the visual representation of the information in the application 4Peaks. Each colored peak corresponds to a specific nucleotide in the sequence. From 4Peaks we were able to extract the entire nucleotide sequence shown for BLAST analysis. The sample sequence most accurately aligned with the *Wnt5b* sequence of *Amblyraja radiata*, the thorny skate, with a similarity of 97%. The sequence data confirmed we had *Wnt5b* DNA and not *Wnt5a* as previously expected.

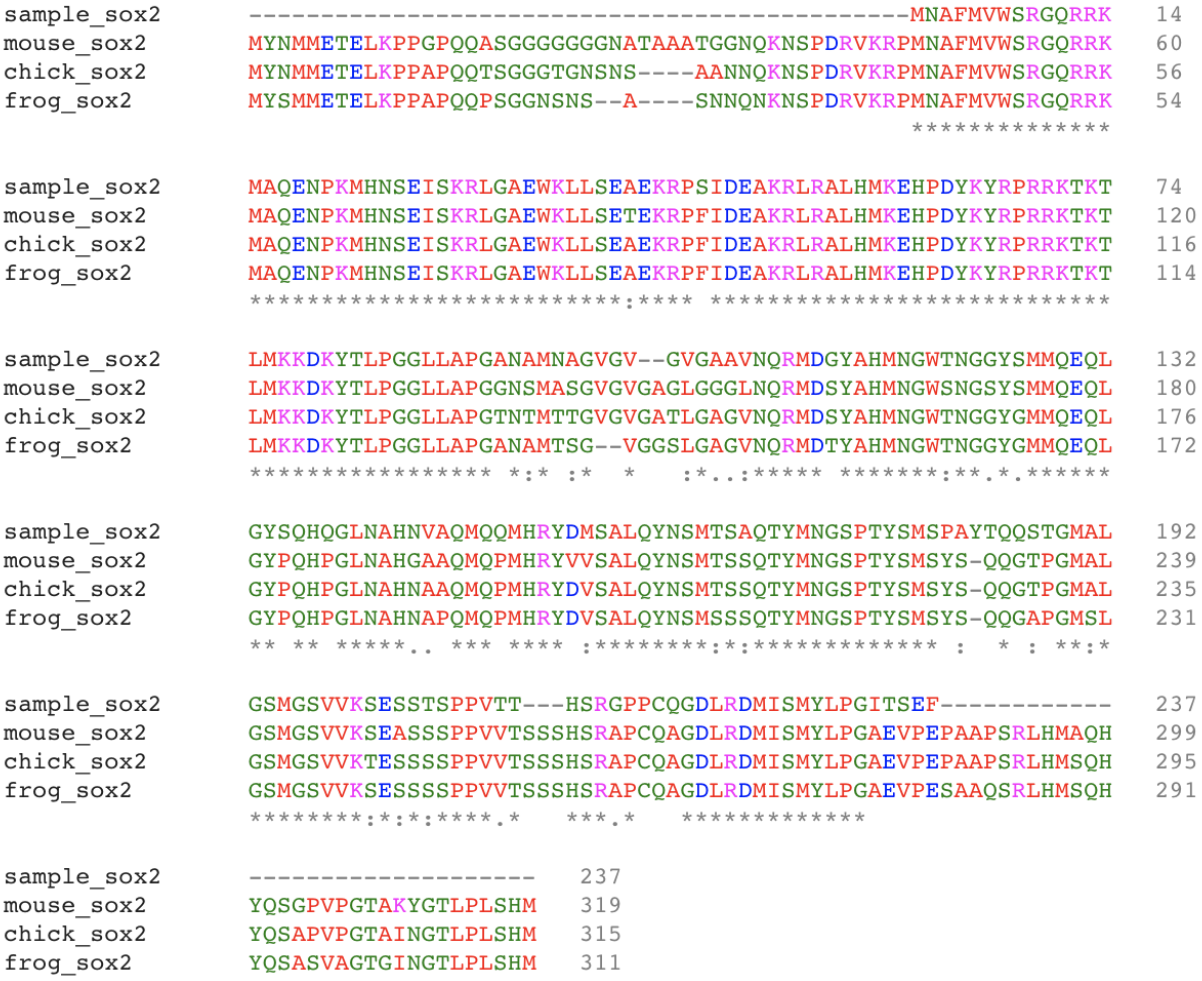

Figure 14. Multiple sequence alignment of our cloned *Sox2* gene fragment translation with multiple species. The sample sequence is 88% identical to the mouse sequence, 84% identical to the chick sequence, and 71% identical to the frog.

Score	Expect	Method	Identities	Positives	Gaps	Frame
239 bits(610)	2e-73	Compositional matrix adjust.	114/118(97%)	114/118(96%)	0/118(0%)	-1

```
Query  354  LVINYNPDIIFKDEENTGADRLMTQRCKDKLNSLAISVMNQWPGVKLRVTEGWDEDGHHS  175
            L  NYNPDIIFKDEENTGADRLMTQRCKDKLNSLAISVMNQWPGVKLRVTEGWDEDGHHS
Sbjct  75   LTPNYNPDIIFKDEENTGADRLMTQRCKDKLNSLAISVMNQWPGVKLRVTEGWDEDGHHS  134

Query  174  EESLHYEGRAVDITTSDRDRSKYGMLARLAVEAGFDLVNYESKAHIHXSVKAENSVAA  1
            EESLHYEGRAVDITTSDRDRSKYGMLARLAVEAGFD VNYESKAHIH SVKAENSVAA
Sbjct  135  EESLHYEGRAVDITTSDRDRSKYGMLARLAVEAGFDWVNYESKAHIHCSVKAENSVAA  192
```

Figure 15. BLAST alignment result for the translated *Shh* DNA sent for sequencing (Query) and the *Shh* sequence provided from the NIH database (Subject) for *L. erinacea*. Highlighted in red are the percent identities and positives, indicating that 97% of the two sequences have identical or conservatively substituted residues. Alignment confirms *Shh* was successfully isolated and cloned from skate.

Gene expression

Results from RNA *in situ* hybridization in chick embryos revealed that at stage 27, *Hoxd12* is expressed in the developing limb buds as previously shown (Figure 10) (Cho et al., 2008). *CdxA* expression was detected in the endoderm of the developing chick gut tube, and appeared at the demarcation point between the stomach and intestinal epithelium through to the cloaca (Figure 10). Initial results for the *in situ* in skate with *Shh* and *Sox2* did not produce definitive staining **(**Figure 11**)**. The procedure was repeated after modifying the proteinase-K treatment and solutions, but we were unable to produce observable results. The *in situ* for *Hoxa13* utilized a proteinase-K treatment of 30 µg/mL for 15 minutes and microscopic analysis revealed successful staining in the endoderm of the rectal gland, cloaca and tip of tail of stage 26 skate embryos (Figure 12).

Discussion

The main goal of the research was to visualize the expression patterns of genes identified for their role in vertebrate gastrointestinal tract development. The little skate serves as an example of a primitive vertebrate that can provide information on vertebrate evolution and developmental processes. The location of a gene expressed in the body reflects the function of that gene in the specification and development of that region. Modification to gene expression locations within different organisms allows us to understand the mechanism behind evolution of characteristic digestive tract organ morphologies, like the spiral intestine in the little skate.

Cdx Genes

The initial results in chick provided distinct staining patterns for c*CdxA. CdxA* belongs to the *Cdx* gene family that functions in intestinal development and homeostasis (Costkun et al., 2011). We therefore expected to visualize *CdxA* expression in the endoderm of the midgut / hindgut. Our results revealed *CdxA* expression in the endoderm of the developing chick gut that marked the beginning of intestinal epithelium (Figure 10). *Cdx2,* a gene of interest for our research in skate, is known to function as an early marker of intestinal fate. Differences in the concentration of *Cdx2* in the developing gut tube endoderm result in the differentiation between the midgut and hindgut, with a high concentration patterning the large intestine and a lesser concentration patterning the small intestine (Gilbert, 2016). We therefore hypothesized that *Cdx2* expression would be strongest in the hindgut endoderm and less prominent in the midgut endoderm. While we were unable to visualize the expression pattern of *Cdx2* in skate, the evidence of *CdxA* marking the beginning of intestinal fate in the developing chick endoderm emphasizes the proposed role of *Cdx* genes on intestinal development. The results strengthen our hypothesis for the expected *Cdx2* expression pattern in skate. Future work to visualize *Cdx2* expression in skate will build upon the understanding and comparison of vertebrate *Cdx* genes in gut development.

Hox Genes

The *Hox* genes are a family of transcription factors that regulate axial patterning (Myers, 2008). *Hoxd12* plays an instructive role in limb development across vertebrates and in terms of gut development expression has been observed in the chick and mouse hindgut (Illig et al, 2013). We hypothesized that the function of *Hoxd12* would be conserved and expression would be localized to the developing limbs and terminal gut. We observed distinct staining in the

developing limb buds, but did not find staining in the gut. Due to the fact that gut staining would be in the endoderm, it is possible that the proteinase-K treatment was not strong enough for the probe to completely infiltrate that germ layer. However, the results are consistent with the idea that although the genes involved in vertebrate gut development are highly conserved, differences in gene function exist within organisms.

We observed the expression of *Hoxa13* in skate. Staining was clearly regionalized to the hindgut, specifically the rectal gland, cloaca, and tip of the tail. The results aligned with our hypothesis that *Hoxa13* would be expressed in the hindgut endoderm in skate, indicating that the function of *Hoxa13* is likely conserved in gut development throughout vertebrate evolution. Our results further emphasize the importance of *Hox* genes in specifying the anterior-posterior axis during development, specifically that *Hox* genes establish demarcations between the major AP axis regions of the gut tube (foregut, midgut, and hindgut). The staining pattern of *Hoxa13* in the hindgut serves as an example for the correlation of expression pattern and gene function in a developing embryo.

Shh, Wnt5a, and Sox2

We based our initial hypotheses for expected gene expression patterns on the predicted role of each gene in gastrointestinal tract development in organisms such as mouse, chick, and human. Although we were unable to successfully visualize expression of *Shh, Wnt5a,* and *Sox2* in skate, each gene was anticipated to play an important role in embryonic gut tube patterning. The genes deserve further study to understand how each gene functions in early embryonic gut tube patterning to define the organs and functional regions of the digestive tract in the little skate.

Table 2. Summary of visible gene expression location detected for the genes in chick and skate.

Chick	***Hoxd12*** Limb buds	***CdxA*** Small / large intestine	
Skate	***Hoxa13*** Rectal gland, cloaca, tip of tail	***Cdx2*** Unsuccessful detection	***Sox2*** Unsuccessful detection

Future Work / Limitations

A large portion of the work to prepare for the *in situ*s included synthesizing the RNA probes from our DNA samples for each gene. At various steps in the process we checked for results using gel electrophoresis and subsequent imaging. One limitation of the research is that result analysis could have been more thorough. There were instances when the band clarity was ambiguous and interpretation therefore unclear. In future work with DNA prep I would repeat the steps until distinct results were visible before moving on in the process. Additionally, each gene sequence has a characteristic base pair length resulting from the PCR product that was inserted in the vector. Following the restriction enzyme digest, the insert is released from the vector and we should be able to visualize the insert at the expected band length for the given gene. Another limitation of the DNA prep was that we did not inspect for specific insert base pair lengths, only the 3kb vector and visual confirmation of another band. We sent the majority of DNA out to confirm the sequence of the insert, but for *Cdx2* we solely relied on the presence of a vector and insert band. In this case, it would have been worthwhile to analyze the band lengths for the expected base pair length of the *Cdx2* PCR product to ensure the product was the gene we cloned. Going forward, I believe implementing a more methodical approach to analyzing the DNA will ensure this portion of the research provides a sure foundation to proceed with the *in situs.*

We experienced trouble producing definitive gene expression patterns in skate. The initial trial of *in situ* in chick was successful, indicating that the protocol and solutions/reagents used in the lab were functioning properly. Through troubleshooting we hypothesized that the initial proteinase-K treatment was not strong enough. We then increased the treatment concentration from 10 ug/mL for 15 minutes to 30 ug/mL for 15 minutes for skate embryos in the *Hoxa13 in situ,* which was the first time we observed a definitive staining pattern in skate. However, when we repeated the experiment with the increased proteinase-K concentration for embryos with *Sox2* and *Cdx2,* we were unable to discern distinct staining. After ruling out proteinase-K as the source of the problem due to its success with the *Hoxa13* embryos, the only difference between the *in situ* trials was then the source of the DNA. The *Hoxa13* RNA probe was transcribed from the EST (expressed sequence tag) form of the DNA as opposed to the other gene sequence DNA that was cut from a plasmid vector. However, due to the fact that our *Sox2* sequence was confirmed through sequencing, it seems unlikely that this difference would be the sole source of the project's shortcomings. In order to move forward with whole mount RNA *in situ* hybridization work in skate, the protocol must be further analyzed and modified to ensure definitive and repeatable results can be obtained.

Once the expression profiles are successfully compiled for the genes identified in gut tube patterning in skate, the information can be supplemented with future research to build on the understanding of the gene's role in development. For example, layering expression pattern results with phenotypic results of a knock-out experiment can provide deeper insight into the molecular role of the gene in developing a functional gastrointestinal tract. Additionally, a thorough developmental profile for each gene will facilitate the comparison of gene function across vertebrate lineages. Identifying discrepancies in the function of conserved genes unveils

information about vertebrate evolution. Specifically, an organism's unique gene expression profile can explain how modifications to the genes that patterned the primitive digestive tract resulted in the unique gastrointestinal morphologies observed throughout vertebrate evolution.

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