Evidence of a rudimentary colon in *Leucoraja erinacea*

By

Alyssa Mae Simeone

****

Submitted in fulfillment
Of the requirements for
Honors in the Department of Biological Sciences

UNION COLLEGE
June, 2011

ABSTRACT

SIMEONE, ALYSSA Evidence of a rudimentary colon in *Leucoraja erinacea*

ADVISOR: Nicole Theodosiou, PhD

During the transition of animals from water onto land, the colon is believed to have evolved as an essential water-absorbing organ in terrestrial vertebrates to prevent desiccation. The class Chondrichthyes, comprised of sharks, rays, and skates, are isotonic to their marine environment, and thus do not require a functional colon. The Chondrichthyes are an excellent organism for developmental and physiological studies in evolutionary context because they have undergone little evolutionary change since their appearance 450 million years ago. Previous histochemical studies demonstrate potential water absorbing properties in the digestive tract of the little skate, *Leucoraja erinacea* (Theodosiou et al., 2007). To further examine the ability of the skate spiral intestine to absorb water, I examined the expression of the water-specific channel protein, aquaporin 4. Aquaporin 4 is expressed in the skate digestive tract with high levels in the epithelium of the distal spiral intestine. In addition, I measured water uptake across the membrane of the spiral intestine directly using physiology experiments. The distal spiral intestine absorbs water at a greater rate than the stomach and proximal intestine, and water uptake was not pressure dependent. Currently, we are examining the conservation of embryonic colon patterning genes by studying the role of *Hoxd13* in developing chick and skate embryos. With this study, we hope to understand the origin of the colon in terrestrial vertebrates.

INTRODUCTION

The digestive tract regulates the processes of ingestion, digestion and the expulsion of waste. Though digestion starts at the mouth and ends at the anus, there is a system of organs that work together to coordinate digestion and the absorption of nutrients. The stomach digests food using acidic enzymes, the small intestine absorbs nutrients for cell growth, and the large intestine functions to absorb electrolytes and water to prevent dehydration (Lacy, 1991; Stevens and Hume, 1995). Thus, each organ of the digestive tract has distinct and essential functions. Studying the colon is important because of its role in fatal diseases. Dysentery is a disease of the colon and is a leading killer in developing countries, resulting in 750,000 deaths a year. There are also 200,000 new cases of colorectal cancer each year. Thus, understanding how the colon develops and how it evolved can give us insights into disease states.

The colon plays a distinct role in the digestive tract to absorb ingested water and salts in order for the body to remain hydrated (Lacy, 1991). The colon absorbs more water per unit of luminal surface area than the small intestine because of differences in the relative length and luminal surface area of the organs (Wang et al., 2000). The colon and large intestine's absorptive activities work against an osmotic gradient to absorb water. While the small intestine has long finger-like villi protruding from the epithelium into the lumen, increasing surface area for nutrient and water absorption, the colon contains no villi and instead has small protrusions in the epithelium caused by muscular contraction (Lacy, 1991; Stevens and Hume, 1995; Roberts et al., 1998). To enhance water uptake in the absence of villi, the colon contains acid mucin goblet cells and water-specific aquaporins.

Goblet cells are present in tissues where water uptake across the epithelium is increased, such as the kidney and colon (Smith, 1936; Filipe, 1979; Lichtenberger, 1995; Lugea et al., 2000). These cells synthesize and secrete glycoproteins, or mucins, to create a protective mucous layer that maintains the luminal epithelium and acts as a barrier against invaders and macromolecules (Filipe, 1979). Goblet cells may be acidic, basic, or neutral, depending on the properties of the glycoproteins produced and the function of the organs they are present in (Oliver and Specian, 1991). Neutral mucin goblet cells, for example, are typically present in the stomach and duodenum to protect the luminal epithelium from the acidic enzymes digesting food (Filipe, 1979). Acid mucin goblet cells are glandular columnar cells typically found within the epithelia of the colon and the respiratory tract. Elevated acidic mucins are found in the colon, supporting their role in promoting water uptake (Filipe, 1979).

In addition to acid mucin goblet cells, the presence of aquaporins can provide insight into water regulation within the gut. Essential for biological processes, aquaporins are a family of integral membrane protein channels that play important roles in animals and plants by helping to regulate water homeostasis in living cells (Wang et al., 2000; Matsuzaki et al., 2004; Lignot et al., 2002). Aquaporins (AQP) facilitate water movement by forming pores within their potassium channels that are embedded in biological membranes. With a size of only 30 kDa per monomer, aquaporins form tetramers with each monomer defining a single pore (Matsuzaki et al., 2004). In addition to transporting water, some aquaporin family members transport small solutes such as urea and glycerol (Matsuzaki et al., 2004). Like acid mucin goblet cells, aquaporins are found in regions of increased water absorption such as the kidney and the colon (Wang et

al., 2000; Matsuzaki et al., 2004; Lignot et al., 2002). There are 13 identified members of the aquaporin family found in the human body; each aquaporin is specific to different regions of the body and some have different functions (Matsuzaki et al., 2004).

Family members AQP 2, 4, and 8 are water-selective and expressed in the colon of certain terrestrial vertebrates, including humans (Wang et al., 2000; Matsuzaki et al., 2004). AQP8 is expressed in proliferating colonic epithelial cells in human tissue, suggesting a role in water reabsorption across the colon membrane. Previous findings show that human AQP8 is specific for water absorption, and is not permeable to urea and glycerol (Matsuzaki et al., 2004). The presence of AQP2 channels in apical membrane of the surface columnar epithelial cells in the distal colon increases the osmotic water permeability of the apical plasma membrane (Matsuzaki et al., 2004). AQP4 plays a role in colonic fluid absorption in humans (Wang et al., 2000). Evidence from AQP4 knockout (-/-) mice implicates water transport across AQP4 channels in colon epithelium facilitates transepithelial osmotic water permeability (Wang et al., 2000).

While understanding the physiology and cellular architecture of the colon is essential in understanding human disease, the colon is also an important organ in the evolution of vertebrates. The colon was essential during the transition of animals from water onto land, as animals required an organ to guard against dehydration and evaporative water loss (Lacy, 1991). The transition of vertebrates to land caused an internal shift in evaporative loss and solute concentration (Lacy, 1991). Land animals consequently evolved a colon, a water-absorbing organ.

Not all animals require a water-absorbing organ, however, and every vertebrate species has some variation in the shapes and functions of their gastrointestinal tract

organs. The skate, a member of the Chondrichthyes, has a different digestive tract structurally and functionally compared to land vertebrates, and has shown very little change over 500 million years (Stevens and Hume, 1995; Hamlett, 1999). Although the cellular structure of the gut, including epithelium submucosa and mucosa, is present in both aquatic and terrestrial animals, Chondrichthyes and terrestrial vertebrates digest and process food differently in their digestive tracts (Stevens and Hume, 1995). The question still remains, when during animal history did the hindgut evolve to obtain water-absorbing properties of the modern colon.

In addition to a structurally and functionally distinct digestive tract, the cartilaginous fish are isotonic to their ocean environment, thus lacking the need to absorb water though the digestive tract to maintain body fluid (Hamlett, 1999). Despite the skate's isotonicity to its natural environment, there is histological evidence of water absorption in regions of the digestive tract of the little skate, *L. erinacea.* This is unexpected and has evolutionary implications because the skate is water dwelling and is understood to transport water through the gills (Janech et al., 2003). One way to look for water absorbing properties is to characterize the presence of acid mucin goblet cells. Strong expression of acid mucin goblet cells were found in the final turn of the distal spiral intestine of *Leucoraja erinacea*, at levels comparable to those in the colon of the mouse, chicken and other terrestrial vertebrates (Theodosiou et al., 2007). The evidence of acid mucins in the skate digestive tract provides a possible evolutionary link to the colon of terrestrial vertebrates, which suggests the skate may have differential water-absorbing properties in the digestive tract.

All vertebrates, no matter how diverse a body plan or digestive tract, share a common evolutionary ancestor (Stevens and Hume, 1995). Because of this, all vertebrate animals share common development programs and a common phylogenic stage in which they all look alike (Gilbert, 2006). After the phylogenic stage, animals diversify into the varying morphologies we see depicted in animals today. Thus, understanding early development and patterning of digestive tract can give us insight into its evolutionary origin.

The digestive tract is initially formed as a single gut tube during embryogenesis. As the gut tube develops, transcription factors, such as *Hox* genes, play a role in regional specialization of the gastrointestinal tract, though the roles of only a few transcription factors have been elucidated (Gilbert, 2006). These transcription factors give rise to the specific forms and functions of the different organs throughout the body system, which differs between species.

The *Hox* gene family patterns the embryonic plan during development (Roberts et al., 1998; McGinnis and Krumlauf, 1992; Grapin-Botton and Melton, 2000; Kawazoe et al., 2002). *Hox* genes are the foundation for anterior-posterior (AP) axis specification, patterning the limb axis, and support evolutionary homologies among all animals. The gastrointestinal tract is specified regionally at an early stage in development. In a large group of vertebrate animals, the endodermal epithelium responds to various mesodermal cues as the endodermal tube forms (Roberts et al., 1998). These regionally specific signals are directed to different parts of the endoderm and allow the gastrointestinal tract to develop into morphologically and functionally distinct regions that become organs with distinct functions. Morphogenesis and differentiation leads to different phenotypes in each of these organs; for example, the outer muscle layer of the colon is drastically

different from that in the small intestine of humans (Lacy, 1991). *Hox* genes are important to understand the genetic component of gastrointestinal development, specifically the colon.

Previous work focusing on patterning genes in the digestive tract of the chick found that that *Hoxd13* gene is mainly expressed in the large intestine and cloaca of the chick. Misexpression of *Hoxd13* in the developing midgut increases acid mucin levels in the small intestine, and transforms the small intestine into large intestine morphology (Roberts et al., 1998). Thus, this gene is implicated in patterning of the colon in the chick.

In my project, I examined the ability of *Leucoraja erinacea* to absorb water in the digestive tract using physiological and molecular approaches. The goal of these experiments was to measure water uptake directly across the membrane of the adult skate spiral intestine, and to examine the expression of water-specific AQP4 throughout the gastrointestinal tract of the skate. Identifying colon specific AQP4 and acidic mucins throughout species in the vertebrate lineage will give us information about the evolutionary origins of the colon. After examining water-absorbing properties throughout the intestine of the skate, I examined the evolutionary conservation of colon-related genes using the chick model. In order to do this, chick embryos were injected with a RCAS-*Hoxd13* viral vector in order to attempt to transform the midgut to hindgut morphology (Roberts et al., 1998). The transformation of gut morphology will enable us to determine how the conservation of *Hoxd13* functions to pattern the colon.

MATERIALS & METHODS

Measuring H_2O Absorption in adult skates

Digestive tracts were harvested from adult skates, *L. erinacea* and briefly flushed through with running marine water. The stomach and spiral intestine were dissected from the skate digestive tract. In addition, the intestine was further divided into proximal and distal halves. Each organ section was filled with Elasmobranch Ringer's solution until a pressure of 1.0 kPa was reached; the ballooned organs were then tied at each end, and measured for length (cm). The intestine or stomach was placed in an aerated stirring bath of Elasmobranch Ringer's solution. Each tissue was weighed every 30 minutes for a total of 3 hours; the tissue was gently wiped with Kimwipes™ before being placed on the mass balance. Observed changes in mass (mg) corresponded to the amount of water being absorbed by either the stomach or intestine over time.

To examine the role of osmotic pressure on water absorption, the pressure of Elasmobranch ringers solution was maintained at 1.0 kPa inside the gut lumen and increased to 3.0 kPa while changes in mass were measured as above, every 30 minutes for 3 hours. This experimental method was adapted from a similar protocol reported on *Anguilla japonica* (Aoki et al., 2003).

The surface area was calculated using the following formula:

$$Folds[(L*W)*2*(\#\ folds)]+(L*W)_{OL}$$

The rate of water absorption was also calculated:

$$(T_0 - T_3 hour)/surfacearea_{total}/3hour.$$

Detection of AQP4 expression in L. erinacea

Sample Preparation/Protein Quantification/Western Blot/Dot Blot

Fresh skate tissue was harvested at Mount Desert Island Biological Laboratory and stored at -80ºC. To prepare the tissue for protein quantification, a small piece of tissue was cut and ground in liquid nitrogen with a mortar and pestle. The ground tissue was homogenized in TG Lysis Buffer (20mM HEPES pH 7.2, 1% Triton-X 100, 10% glycerol) using a polytron homogenizer, and centrifuged at 12,000g at 4ºC for 10 minutes. The supernatant lysate was transferred to a fresh pre-chilled eppendorf tube and stored at -80ºC. Protein lysates were generated from the stomach, proximal and distal intestines, cloaca and rectal gland of the skate digestive tract.

A bovine serum albumin (BSA) assay was performed to quantify the protein in the skate tissue. BSA standards were prepared according to the Thermo Scientific Pierce® BCA Protein Assay Kit Instructions. The microplate was loaded with the BSA standards and skate samples, followed by the addition of the BCA Working Reagent, and a 30-minute incubation at 37ºC.

A 4-20 % gradient Bio-Rad acrylamide gel was prepared. A 15 µl ladder was used, and rat kidney extract was used as a control (Millipore). Lysis buffer containing protein was loaded with 2x loading dye. Gels were run at 100 V for the first 10 minutes, then increased to no higher than 130 V for 1 hour. When electrophoresis completed, the gel was removed and placed in a dish filled with transfer buffer, and gently rocked for 20 minutes. Six pieces of transfer paper and 1 piece of membrane were cut into 3.25'' x 2'' pieces. Three pieces of western blot paper were soaked in transfer buffer and layed flat on the western transfer machine. The membrane was briefly soaked in methanol

followed by transfer buffer, then placed on top of the blot paper. The gel was layed over the membrane and trimmed to the exact size of the paper using a razor blade. Three more pieces of western blot paper were soaked in transfer buffer and placed on top of the gel. The western transfer machine was run at 15V for 20 minutes, after which the membrane was blocked in 5% milk solution (5g dry milk and 100mL TBST) for 1 hour at room temperature. After four three-minute washes in TBST, the membrane was incubated in Anti-aquaporin 4 (rabbit polyclonal) (Millipore) overnight in 4ºC.

After four four-minute washes in TBST, the membrane incubated in 2º antibody, HRP goat anti rabbit (1:2500) for 1 hour at room temperature. The membrane was washed three times, 4 minutes each, in TBS, then was developed with Thermo Scientific chemiluminescent substract, peroxide solution and enhancer solution. A Fuji camera was used to detect presence of aquaporin 4 on the membrane.

For Dot Blot Analysis a dot of 50 µg of each desired tissue was placed on 2 nitrocellulose membranes and left to dry completely. The membranes were blocked in 5% milk/TBST for 1 hour at RT while gently shaking, then incubated in 1º antibody for 2 hours at RT while gently shaking (1:250 and 1:500). After three 10-minutes washes in TBST, the membranes were incubated in HRP goat anti rabbit at 1:1000 in 5% milk for 1 hour. The membranes were washed three times for 10 minutes, then developed using a Fuji camera.

Paraffin Embedding

In order to detect AQP4 expression in tissue, digestive tracts from *L. erinacea* embryonic day 60 was dissected into different regions (stomach, proximal intestine, mid intestine, distal intestine, hindgut, and rectal gland), fixed in 4% paraformaldehyde, and

dehydrated in an ethyl alcohol (EtOH) series (25%, 50%, 75%, 95%, each for 15 minutes, and two 30 minute washes in 100% EtOH). The tissues were transferred to glass scintillation vials, and washed twice for 5 minutes in xylene. After clearing in xylene, the tissue was incubated in paraffin at 50 ºC, changing the paraffin a few times before incubating overnight in the oven. Tissue was transferred to cassettes and submerged in a vacuum chamber with paraffin for 1-2 hours at 58 ºC. The tissue was embedded and cooled on an ice block for 5 hours, then stored at 4 ºC.

Immunohistochemistry

Paraffin-embedded tissue was cut into 6 µm sections and prepared for biotinylated antibody staining by melting the sections and then cooled to room temperature.

The slides were de-paraffinized in xylene, hydrated in a reverse EtOH series and washed in PBS (phosphate buffer saline). The sections were bleached in 0.5 % H_2O_2 for 30 minutes, washed 3 times in PBS and post-fixed in 4 % PFA for 30 minutes. To enhance the staining intensity of the antibody slides were washed in sodium citrate buffer (10mM Sodium Citrate, 0.05 % Tween 20, pH 6.0). The slides were incubated in 95-100 ºC sodium citrate buffer for 20 minutes and allowed to cool for 20 minutes. After washing in PBT (PBS, 0.1% Tween 20), tissue sections blocked in 10 % horse serum for 30 minutes. Slides were incubated overnight with 3C2 aqp4 antibody at 1:250 in 1 % horse serum overnight at 4ºC while shaking. After 3 PBT washes, the slides washed in 5 % horse serum/PBT and 1 % horse serum/PBT, respectively, each for 10 minutes. Next, the slides incubated in HRP goat anti rabbit 2º antibody at 1:250 in 1 % horse serum for 30 minutes at room temperature. After 3 PBT washes, slides were incubated in a ready to use Vectashield AB mix (Vector Labs), followed by PBT and PBS washes. The slides

were developed with DAB mix for no more than 20 minutes, and were then placed in distilled H_2O before dehydrating in an EtOH series ending in xylene. Slides were mounted with DPX, and tissue-specific expression was observed using a Nikon Eclipse E600 microscope.

Viral misexpression of cHoxd13

cHoxd13 misexpression in midgut mesoderm: Virus Injection

A small window was cut in the top of the eggshell to expose the embryo and covered with tape, followed by incubation at 39ºC. The *cHoxd13* transgene virus was injected into the intercoelomic space of the embryos, and then embryos were left to incubate until harvesting on embryonic day (e) 9 and e18. Whole mount RNA *in situ*'s were performed on e9 embryos to look for viral infection (pictures not shown). Chicks left to incubate until e18 were harvested, then the gastrointestinal tracts were dissected out, embedded in paraffin, and cut into 8µm sections. Adjacent sections were mounted on adjacent slides, and alternating slides were stained for cell morphology, nuclei, and virus detection using hematoxylin/eosin, alcian blue/nuclear fast red and 3C2 staining, respectively.

Hematoxylin and Eosin

Slides were de-paraffinzed in xylene, then hydrated in a series of 100% ethanol washes and deionized water. Slides were stained with Mayer's Hematoxylin for 2 minutes, rinsed under running warm tap water until blue, and washed in dH_2O. After 20 dips in 70% ethanol, the slides were stained with Eosin-phloxine for 30 seconds then hydrated in 3 20-dip washes of 100% ethanol and xylene. Slides were mounted with DPX mounting solution and staining was observed using a Nikon microscope.

Alcian Blue/Nuclear Fast Red

Slides were de-paraffinized in xylene then hydrated in a reverse ethanol series. After rinsing in dH_2O, slides were stained in Alcian Blue Solution for 5 minutes, and rinsed in tap water for 3 minutes. Slides were rinsed in distilled water before staining in Nuclear Fast Red for 10 minutes, followed by a rinse in tap water for 3 minutes. Slides were rinsed again in distilled water, dehydrated in an ethanol series and xylene. Slides were mounted with DPX solution and observed using a Nikon Eclipse E600 microscope.

Virus Detection

Slides were de-paraffinized in xylene then hydrated in a reverse ethanol series. After a wash in PBS, the slides were incubated in 0.3% H_2O_2 /PBS for 30 minutes, followed by another wash in PBS. Slides were then incubated in 4% PFA/PBS for 30 minutes followed by 3 PBS washes. After a 30 minute incubation in MST, slides were incubated overnight at 4ºC in 1:5 Mouse anti 3C2 in MST. After 2 PBT washes, the slides were washed with 5% HS/PBT and 1% HS/PBT, each for 10 minutes. The slides then incubated in biotinylated horse-anti-mouse IgG at 1:250 in 1% HS/PBT for 30 minutes at room temperature, followed by 3 PBT washes. The slides were then incubated in AB mix for 30 minutes followed by 2 PBT washes and one wash in PBS. The slides were developed with DAB for 2-5 minutes then immediately washed in PBT followed by a dehydration series. The slides were mounted in DPX, left to dry, then tissue-specific staining of the virus was observed using a Nikon microscope.

Skate Hoxd13 ortholog plasmid

The skate DNA containing the *Hoxd13* gene was inserted into a vector plasmid with a 4:1 ratio of inserted DNA to vector plasmid. The 3’ end was cut with Sca-R1 and

the 5’ end was cut with Xba-Sma. Polymerase Chain Reaction was run for the 3’ end from the full-length gene. Primers ScaI and EcoRI were used. Sma1 and EcoRI were used to cut blues script containing the 5’ end of the gene. A ligation was performed to attach the 3’ and 5’ ends, which was confirmed with sequence analysis. We took the full-length gene and put it into pSlax for shuttling it to RCAS vector. pSlax was cut with EcoRI and NcoI, then cut with ClaI and put into the RCAS vector. Mini preps were performed using Qiagen kits.

RESULTS

Water absorption in the skate digestive tract

Previous work showing the presence of acid mucins in the distal spiral intestine of the skate digestive tract suggests that the spiral intestine of the skate may have water-absorbing properties. To determine whether the skate spiral intestine absorbs water I did physiology experiments to measure water uptake directly across the intestinal epithelium of *L. erinacea*.

The data presented show that the skate spiral intestine does in fact absorb water. Water absorption, measured as Δ mg water per cm of tissue every 30 minutes over 3 hours, was graphed over time for both the stomach and spiral intestine of the adult skate at 1.0 kPa (Figure 1). There was a constant increase in water uptake over time across the membrane of the intestine and the stomach. Water absorption across the stomach epithelium was significantly less than the spiral intestine (Figure 1). The graph suggests statistical significance in the amount of water absorbed in the stomach and spiral intestine because the standard deviation bars do not overlap (Figure 1).

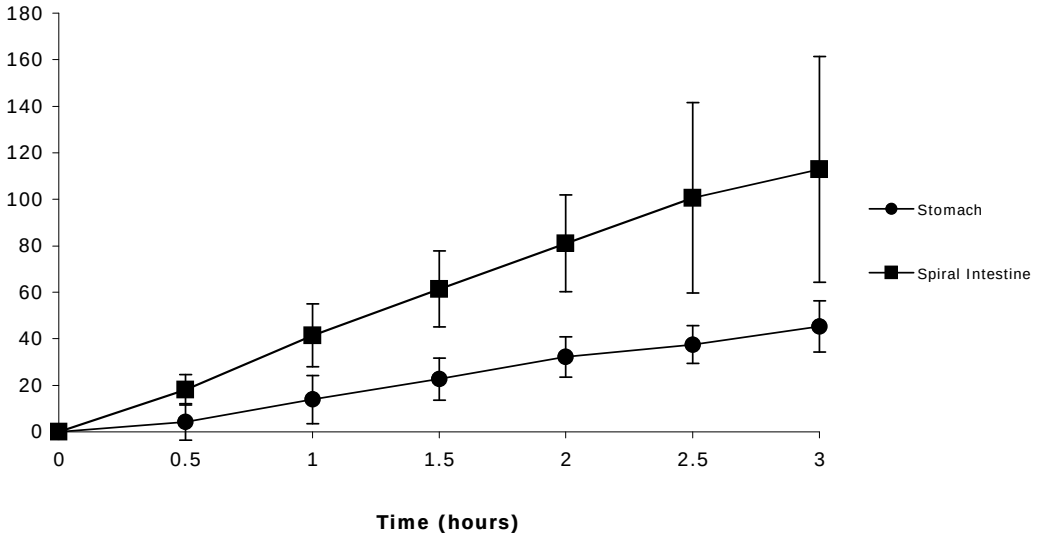

Figure 1. Average Water Absorbed (mg/cm) vs. Time (hours). Change in weight was measured over 30 min. time intervals in the stomach and spiral intestine of the skate, *Leucoraja erinacea*.

After measuring water uptake in the stomach and spiral intestine, we divided the spiral intestine into its proximal and distal ends to identify any differences in water absorption in the two regions. (Figure 2). Both parts of the spiral intestine absorbed significantly more water than the stomach (Figure 2). There was no significant difference in water absorption between the proximal and distal ends of the spiral intestine of *L. erinacea* (Figure 2).

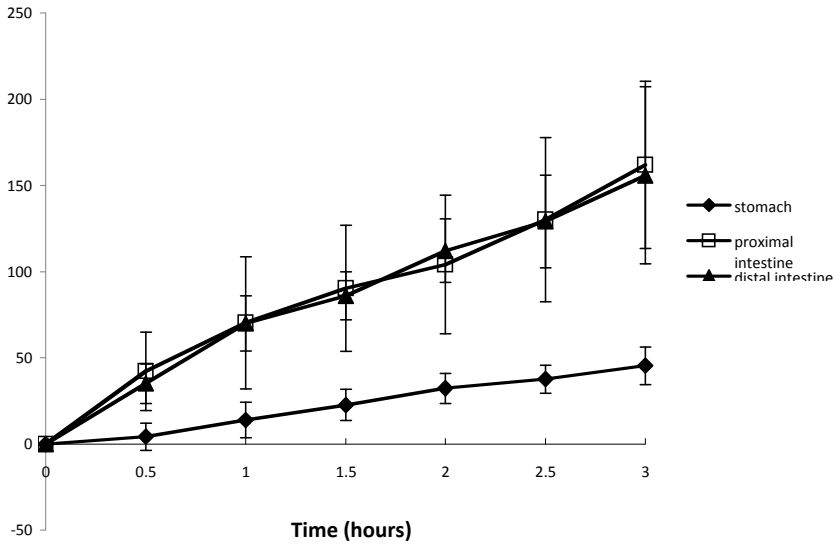

Figure 2. Average mg of water/cm vs. Time. Water absorption in the stomach, proximal and distal spiral intestines of *L. erinacea* at 1.0 kPa.

To determine if hydrostatic pressure inside the lumen played a role in water absorption, the experiment was repeated with the spiral intestine at a pressure of 3.0 kPa. Absorption in both the stomach and the spiral intestine were not pressure-dependent because there was no statistical significance in the rates between each pressure (Figure 3).

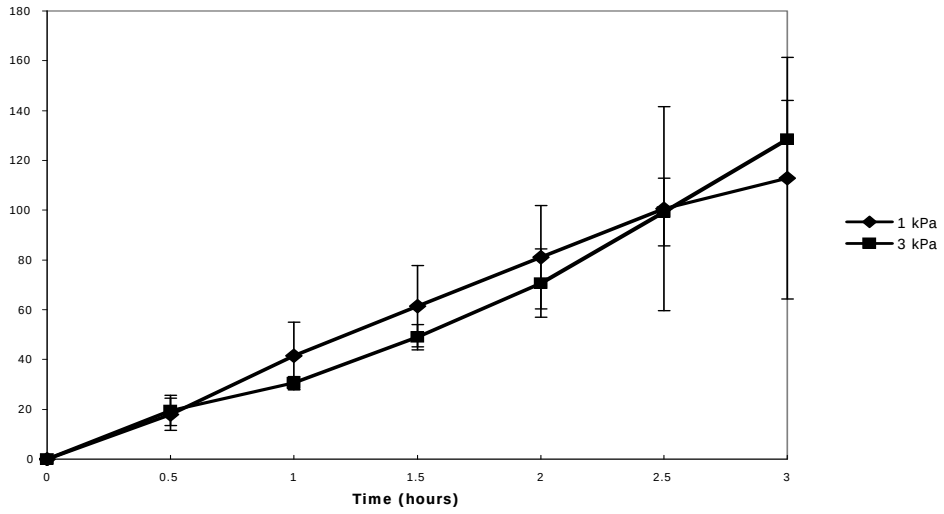

Figure 3. Water Absorption measured at 1.0 kPa and 3.0 kPa. The water absorption across the membrane was determined by measuring the change in weight of the organs every 30 minutes over 3 hours at 1.0 kPa and 3.0 kPa.

I calculated the rate of water absorption in each organ by determining the surface area of both the stomach and spiral intestine (Table 1). The surface area formula used is shown in the *Methods*. The stomach had a 2-fold greater rate of water absorption (P=0.02) than the spiral intestine (Table 1).

	Stomach	***Spiral Intestine***
Average Length (cm)	*6.2*	*7.3*
Average Surface Area (cm^2)	*25.4*	*140*
Average Rate of H_2O Absorption @ 1.0 kPa ($mg/cm^2/hr$)	*4.1*	*2.0*
ST DEV	*0.9*	*0.4*
P value	*0.02*	

Table 1. Average rate of water uptake across the stomach and spiral intestine. The average rate of water absorption was calculated using the length and surface area values and the equation shown in *Methods*. The skate stomach has a greater rate of water absorption than the spiral intestine at 1.0 kPa.

The rate of water absorbed at 1.0 kPa was calculated for the proximal and distal ends of the skate spiral intestine separately. The distal intestine had a 3-fold greater rate of water absorption at 1.0 kPa than the proximal intestine (Table 2).

	Proximal Intestine	***Distal Intestine***
Average Length (cm)	*4.3*	*2.4*
Average Surface Area (cm^2)	*57*	*54*
Average Rate of H_2O Absorption @ 1.0 kPa ($mg/cm^2/hr$)	*2.5*	*7.4*
ST DEV	*1.9*	*1.1*
P value	*0.01*	

Table 2. Average rate of water uptake in the proximal and distal halves of the spiral intestine of *Leucoraja erinacea.* The rate of water absorption was calculated using the length and surface area values for each tissue. There is no significant difference in the rate of water absorption between the proximal and distal ends of the skate spiral intestine.

AQP4 expression in the skate digestive tract

Antibody specificity was confirmed using dot blot analysis. Rat brain was used as a positive control, and dots correlating to AQP4 were present in the skate tissue. There was a stronger signal in the rectal gland, cloaca and distal intestine (Figure 4).

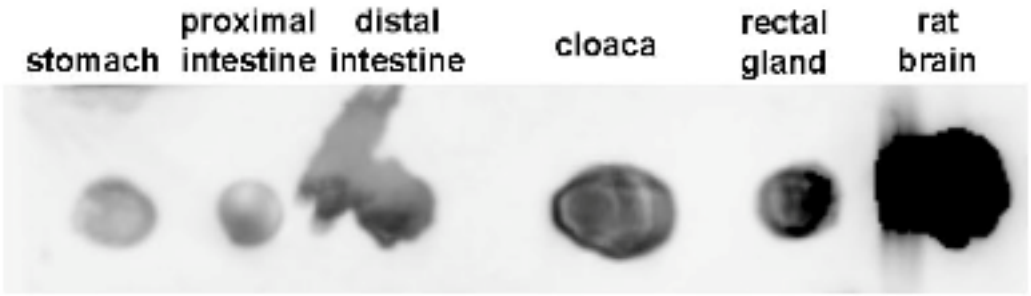

Figure 4. Dot Blot Analysis of AQP4 expression in *Leucoraja erinacea* digestive tract tissue. Rat brain was used as a positive control.

With physiological results that the skate stomach and spiral intestine absorb water across their epithelia, and evidence that water uptake is not pressure dependent, we performed immunohistochemistry to examine the presence of the water-specific protein channel, AQP4, in the skate.

Evidence of weak AQP4 expression in the skate digestive tract was found in the stomach epithelium (Fig. 5 A). In the proximal intestine, expression of AQP4 was present in the crypts of the villi and in the muscle (Fig. 4 B). Strong expression of AQP4 was found in the distal intestine epithelium (Fig. 5 C). The cloaca showed AQP4 expression in the epithelium and muscle (Figure 5 D). No submucosal staining was present in any organ (Fig. 5 A,B,C,D). Negative controls in the absence of primary antibody contained no staining (Figure 5 A',B', C', D').

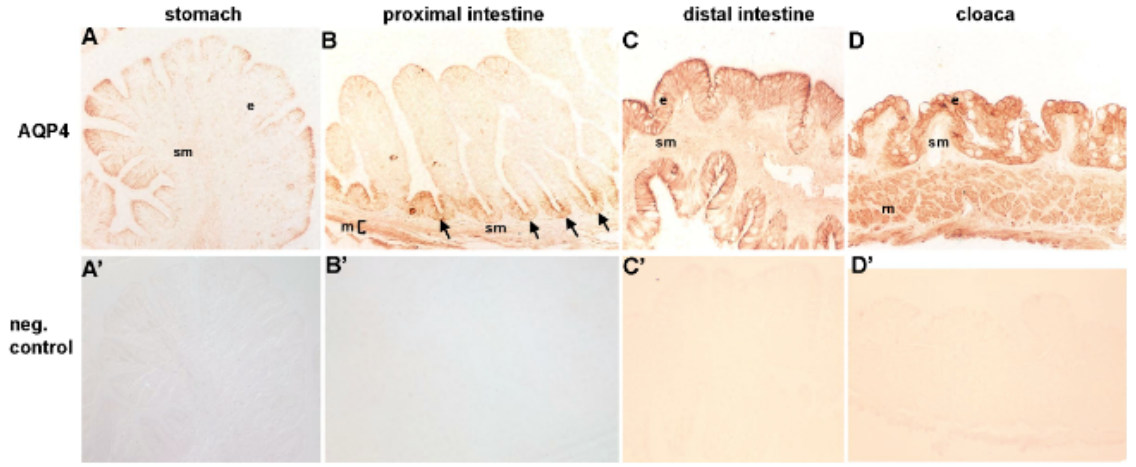

Figure 5. AQP4 expression in *Leucoraja erinacea* digestive tract tissue. (A) Faint expression of AQP4 in the stomach epithelium. (B) Strong expression of AQP4 in the crypts of the proximal intestine. (C) Strong expression of AQP4 in the distal intestine epithelium. (D) AQP4 expression in the skate cloaca epithelium and muscle. (A', B', C', D') Negative controls in corresponding tissue. e, epithelium; sm, submucosa; m, muscle

With this work, we have demonstrated cross-reactivity of polyclonal Ab and the presence of AQP4 in the intestine of *L. erinacea.*

Viral Misexpression

In order to determine if *Hoxd13* patterns the large intestine, chick embryos were injected with an RCAS *cHoxd13* viral vector. Normal phenotypes were observed in 4 of the virus-injected guts, while outwardly distinctive phenotypes were present in the other 3 guts. Two chick embryos were not injected in order to compare results to a wildtype. Digestive tracts of injected embryos showed abnormal phenotypes (Figure 6B). The gut tube in the viral infected embryos were discolored, disorganized and had a shorter gut tube length.

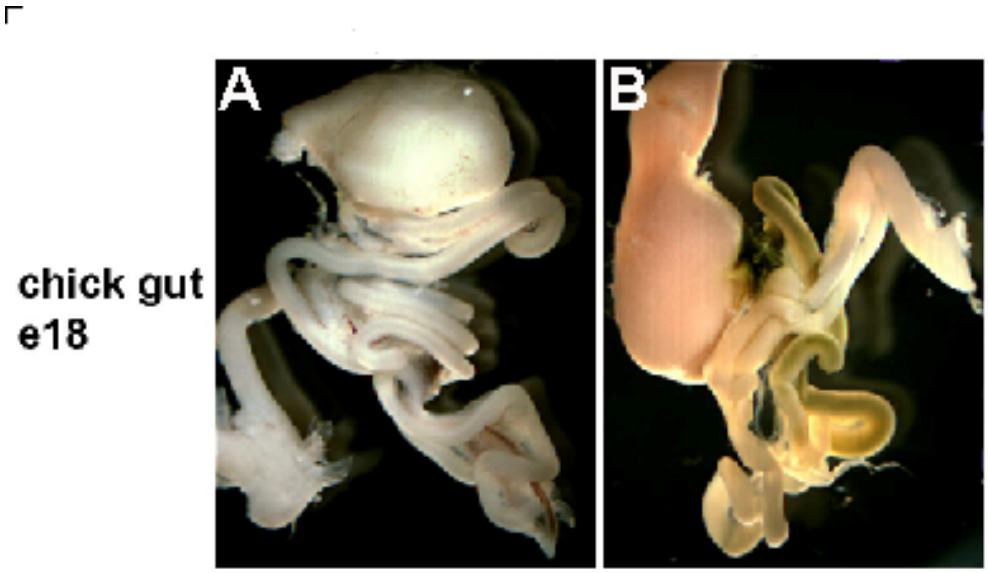

Figure 6. Digestive tracts of infected chick embryos. (A) Normal morphology of the chick digestive tract. (B) This digestive tract was dissected from a chick embryo that was infected with c*Hoxd13*-expressing virus.

Upon histological analysis, the gizzards of the *Hoxd13*-expressing chick embryos appeared to have disorganized tissue layers (Figure 7). The gizzard epithelium was filled with large vacuole-like structures and the submucosa was filled with long, finger-like vacuole air sacs (Figure 7). The gizzards also showed viral infection in the mesoderm and submucosa (Figure 7). The chick embryos that showed a phenotype after injection also showed slightly flattened microvilli in the gizzard as compared to the wildtype (Figure 7).

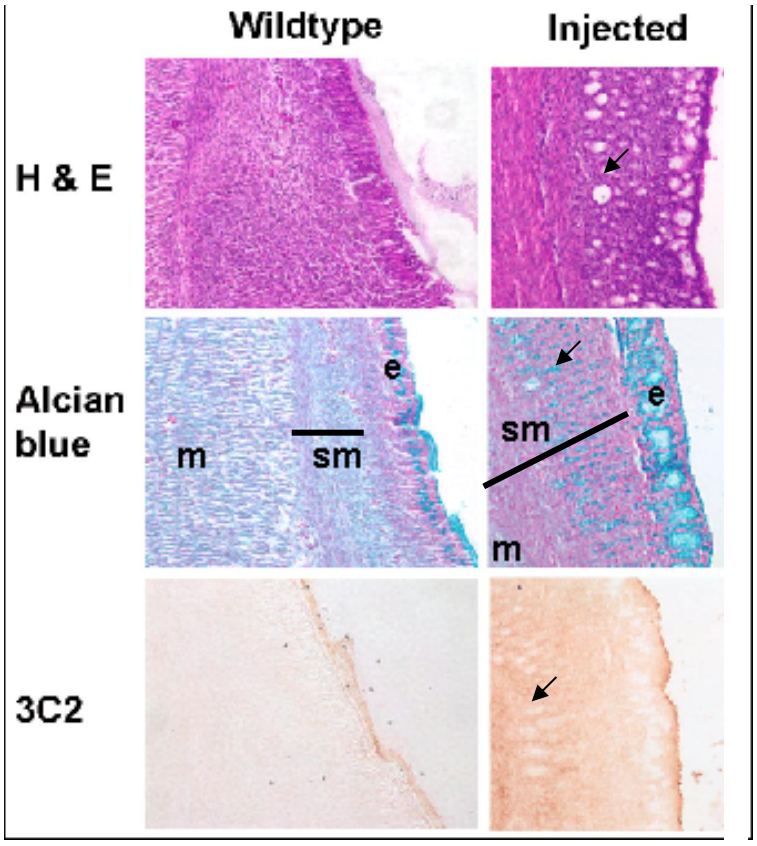

Figure 7. Gizzards misexpressing *cHoxd13* have large vacuoles and ambiguous tissue layers. The wildtype images show organized and distinct tissue layers. Infected gizzards show large vacuoles throughout the epithelium and the submucosa, as pointed out by the black arrows for each stain. The infected embryos show disorganized tissue layers, as best exemplified in the H&E staining. The 3C2 staining confirms that the gizzard was infected with the virus (brown staining). e, epithelium; sm, submucosa; m, muscle.

Misexpression of *cHoxd13* showed a large variation in duodenum epithelium appearance. Phenotypes included flattened villi while some had globular-like villi that were filled with red blood cells (Figure 8). The villi in the duodenum of the injected chick embryos looked very abnormal – red blood cells, as depicted by the red staining, extended into the villi instead of into the capillaries in the submucosa layer (Figure 8). In addition, some villi in the duodenum of the injected chick embryos looked like those found in the rectum, they were flattened.

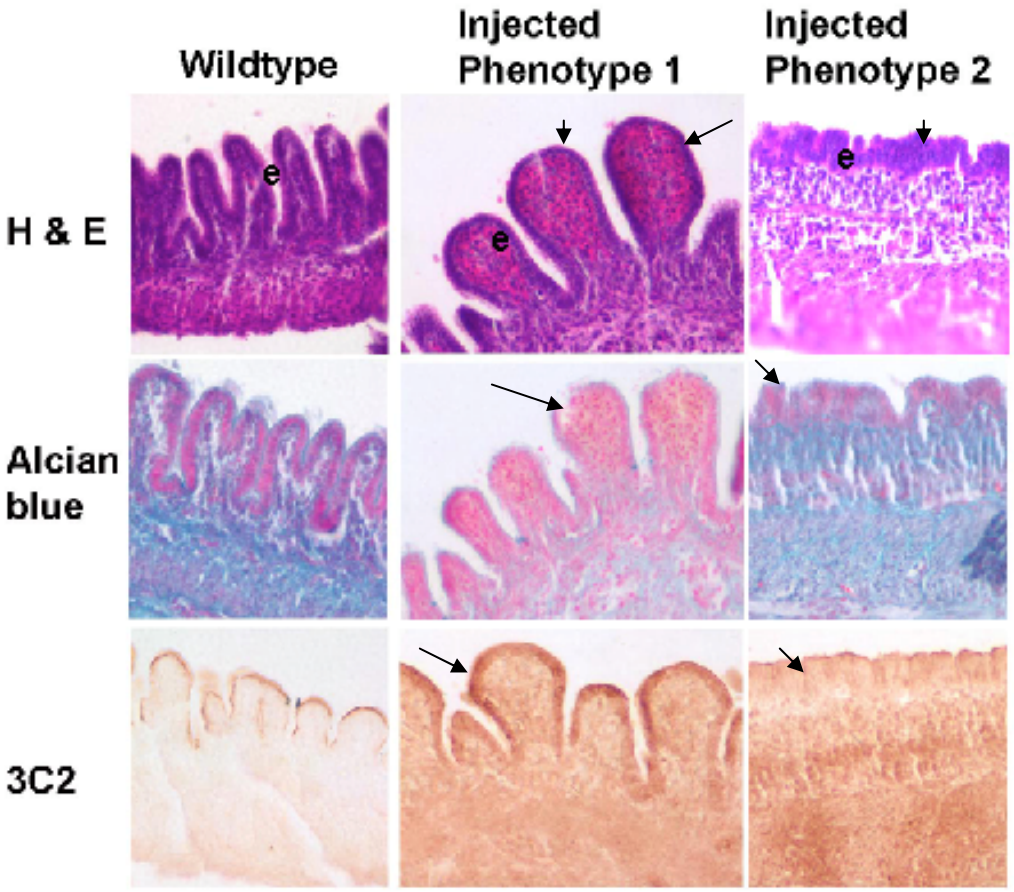

Figure 8. Morphology in the small intestine upon viral misexpression of *cHoxd13*. The wildtype images show a normal small intestine morphology in the chick digestive tract. The first observed phenotype upon *cHoxd13* misexpression was large, bulbous villi filled with red blood cells, pointed out with arrows. These images show a phenotype change in the chick small intestine. Upon infection with the RCAS-*cHoxd13* viral vector, the villi protruding into the lumen became flattened and short, as represented by the arrows. e, epithelium.

As mentioned earlier, some of the injected chick embryos showed a distinct phenotype change, while others did not. We called these the P and "–" phenotypes. The ceca in the "–" phenotype guts showed lots of acid mucins (Figure 9), but no finger like villi as was expected. The P phenotype guts also showed acid mucins, and had flattened villi. More generally, the ceca in the injected chick embryos showed a thick epithelium and disorganized embryonic layers (Figure 9).

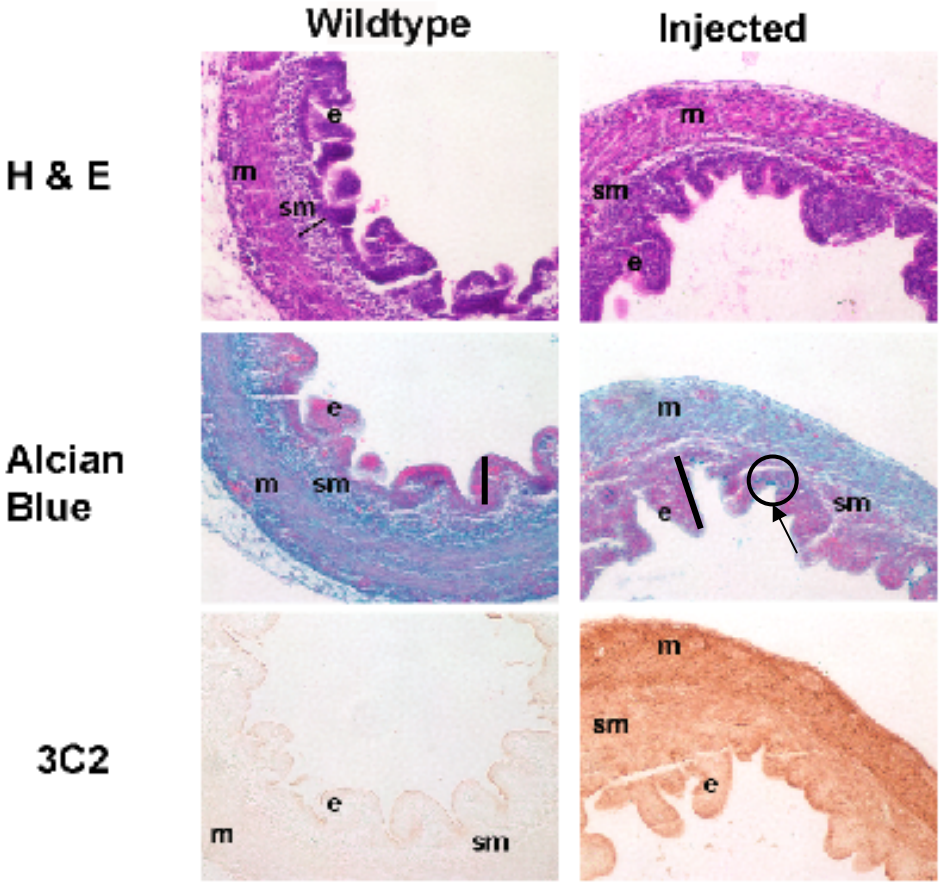

Figure 9. Morphology in the ceca upon viral misexpression of c*Hoxd13*. The wildtype images show the normal morphology of the ceca in the chick embryo. Upon infection with virus, we observed an increase in the number of acidic mucins in the crypts. The acid mucins are the blue dots in the alcian blue staining, and are pointed out with arrows and circled. We also saw a thicker epithelium in the ceca upon c*Hoxd13* misexpression. e, epithelium; sm, submucosa; m, muscle.

DISCUSSION

In this study, our focus was to better understand the origin of the colon in terrestrial vertebrates, and its evolution and development in animals. During the transition of animals from water onto land, the colon developed to prevent dehydration and evaporative water loss.

The elasmobranch digestive tract absorbs water

We set out to determine if the digestive tract of the little skate, specifically the distal spiral intestine, absorbs water by measuring the rate of water uptake directly across the membrane. Our study found that the skate does in fact absorb water across its membrane, which supports our belief that cartilaginous marine fish contain a rudimentary colon. Our physiology work confirms active water transport across the epithelium in the digestive tract of *L. erinacea* to compliment the evidence of acid mucin goblet cells in the skate digestive tract. These results are unexpected because as a member of the Chondrichythes, the skate is isotonic to its aquatic environment, and thus does not need to absorb water through their digestive tract to maintain their osmolarity

Distal spiral intestine of Leucoraja erinacea has water-absorbing properties

The presence of acid mucin goblet cells and the pressure-independent mechanism of water transport across the spiral intestine of *L. erinacea* suggests that passive transport proteins such as aquaporins may also be present in this region of the skate. We used immunohistochemistry to look for evidence of these embedded water channels.

Our results demonstrated that AQP4 is expressed strongly in the distal end of the skate spiral intestine. This suggests that the skate spiral intestine has water-absorbing properties, which supports our hypothesis that cartilaginous marine fish contain a

rudimentary colon; AQP4 is present in the colonic epithelium of mice and facilitates transepithelial osmotic water permeability (Wang et al., 2000).

Looking for the presence of other water-specific aquaporins in the skate digestive tract would support that the spiral intestine actually has water-absorbing properties. The rat, a terrestrial vertebrate, also contains AQP3 and AQP8 in the colonic epithelium, suggesting their role in facilitating water permeability in the colon (Wang et al., 2000). It has been suggested that AQP3 provides water for the epithelial cells that are exposed to harsh environments, such as those facing feces, and is thought to be present in the rectum and distal colon (Matsuzaki et al., 2004).

Since we do not expect the skate to have a water-absorbing organ because of the skates' iso-osmolarity to their ocean environment, the question remains, how did the colon develop, and what genes are responsible?

Misexpressed cHoxd13 alters small intestine morphology in the chick

The presence of *Hoxd13* gene in the same location in both the skate and the chick suggests that it may have the same function in these animals as well (Theodosiou et al., 2007). We set out to replicate the experiment performed by Roberts et al. (1998) to validate that *Hoxd13* is a colon-patterning gene. Even though we did observe morphological changes in the small intestine of the chick upon infection with virus, no final conclusions about the suggestive role of c*Hoxd13* can be made. Acid mucin goblet cells need to be counted in each digestive tract organ in the infected chick embryos, as acid mucin goblet cells are found in regions of increased water uptake and are typically present in large amounts in the colon. The large intestine has flattened, short microvilli and no villi, which is one of the phenotypes we observed in the chick small intestine upon

viral infection. If the amount of acid mucin goblet cells in the infected small intestine are at levels comparable to those in the large intestine or colon of terrestrial vertebrates, we would have some further insight as to the role of *Hoxd13* during development.

In order to determine if the role of *Hoxd13* is conserved along the vertebrate lineage, we will observe any morphology changes in the chick digestive tract upon injection with the skate ortholog of the gene, Le*Hoxd13*. If Le*Hoxd13* can replace the chick *Hoxd13*, this will suggest a conserved role of *Hoxd13* and will give us insight as to when and where the colon originated in animals.

While the main focus of our genetic studies has been *Hoxd13*, it has been suggested that *Hox* gene expression may be in response to a *Shh* endodermal signal because mis-expression of the *sonic hedgehog (Shh)* gene was shown to activate ectopic expression of *Hox* genes in the hindgut mesoderm (Roberts et al., 1998). Therefore, *Shh* may be a gene of interest to study in the future to observe development patterns of the digestive tract.

The majority of our work is based on the knowledge that the main function of the colon is to absorb water (Lacy, 1991; Wang et al., 2000). Once this work is completed, it may be useful to use other properties and characteristics of the vertebrate colon to determine how and where it originated. For example, studies show that the jejunum and colon have different amounts of the vimentin gene and enzyme specific activities in bovine epithelia; jejunum has a higher specific activity (Rusu et al., 2005). Observing differences in enzyme activity throughout the digestive tract could give us insight as to how the colon is functioning. If we find a higher activity of a certain enzyme we may be able to hypothesize a method of water transversion across the epithelial wall.

This study has discovered water-absorbing properties in the digestive tract of the skate, *Leucoraja erinacea*, specifically in the distal spiral intestine, due to its ability to transverse water across the epithelium, and because of the presence of water-specific AQP4 and acid mucin goblet cells. Since the colon's main function is to absorb water, our findings suggest that the skate may contain a rudimentary colon. Understanding the colon will enable us to better understand disease states that originate in the colon.

REFERENCES

1. Lacy, Eric R. "Functional Morphology of the Large Intestine." *Handbook of Physiology*. 121-94. 1991. Print.

2. Stevens, C. Edward and Hume, Ian D. "Comparative Physiology of the Vertebrate Digestive System: Second Edition." Cambridge University Press: New York, NY. 1995.

3. "dysentery." The American Heritage® Medical Dictionary. 2007. Houghton Mifflin Company http://medical-dictionary.thefreedictionary.com/dysentery

4. "What Are the Key Statistics about Colorectal Cancer?" *Learn About Cancer*. American Cancer Society, 2 Mar. 2011. Web.

5. Wang K. S., Ma T., Filiz F., Verkman A. S., Bastidas J. A. "Colon water transport in transgenic mice lacking aquaporin-4 water channels." *Am J Physiol Gastrointest Liver Physiol* 279 (2000): G463-G470.

6. Roberts, Drucilla J., Devyn M. Smith, Deborah J. Goff, and Clifford J. Tabin. "Epithelial-mesenchymal Signaling during the Regionalization of the Chick Gut." *Development* 125 (1998): 2791-801. Print.

7. Smith H. "The retention and physiological role of urea in the Elasmobranchii" *Biol Rev* 11 (1936): 49-82.

8. Filipe, M. I. "Mucins In The Human Gastrointestinal Epithelium: A Review." *Invest. Cell Pathol.* 2 (1979): 195-216. Print.

9. Lichtenberger LM. "The hydrophobic barrier properties of gastrointestinal mucins." *Annu Rev Physiol* 57 (1995): 565-583.

10. Lugea A, Salas A, Casalot J, Guarner F, Malagelada J-R. "Surface hydrophobicity of the rat colonic mucosa is a defensive barrier against macromolecules and toxins." *Gut* 46 (2000): 515-521.

11. Oliver M.G., Specian R.D. "Functional biology of intestinal goblet cells." *Am J Physiol Cell Physiol* 260 (1991).

12. Matsuzaki T., Tajika Y., Ablimit A., Aoki T., Hagiwara H., Takata K. "Aquaporins in the digestive system." *Med Electron Microsc* 37 (2004): 71-80.

13. Lignot J. H., Cutler C. P., Hazon N., Cramb G. "Immunolocalisation of aquaporin 3 in the gill and the gastrointestinal tract of the European eel *Anguilla anguilla* (L.)." *J Exp Bio* 205 (2002): 2653-2663.

14. Hamlett, William C. "Sharks, Skates, and Rays: The Biology of Elasmobranch Fishes." Johns Hopkins University Press: Baltimore, MD. 1999.

15. Janech M, Fitzgibbon W, Chen R, Nowak M, Miller D, Paul R, Ploth D. "Molecular and functional characterization of a urea transporter from the kidney of the Atlantic stingray." *Am J Renal Physiol* 284 (2003): f996-f1005.

16. Theodosiou NA, Hall DA, Jowdry AL. 2007. Comparison of acid mucin goblet cell distribution and *Hox13* expression patterns in the developing vertebrate digestive tract. *J. Exp. Zool. (Mol. Dev. Evol.)* 308B: 442-453.

17. Gilbert S F. "Developmental Biology: Eighth Edition." Sinauer Associates, Inc.: Sunderland, MA. 2006.

18. McGinnis W, Krumlauf R. "Homeobox genes and axial patterning." *Cell* 68 (1992): 283-302.

19. Grapin-Botton A, Melton DA. "Endoderm development from patterning to organogenesis." *Trends Genet* 16 (2000): 124-130.

20. Kawazoe Y, Sekimoto T, Araki M, Takagi K, Araki K, Yamamura K. "Region-specific gastrointestinal Hox code during murine embryonal gut develoment." *Dev Growth Differ* 44 (2002): 77-84.

21. Aoki M, Kaneko T, Katoh F, Hasegawa S, Tsutsui N, Aida K. "Intestinal water absorption through aquaporin 1 expressed in the apical membrane of mucosal epithelial cells in seawater-adapted Japanese eel." *J Exp Biol* 206 (2003): 3495-3505.

22. Rusu D, Loret S, Peulen O, Mainil J, Dandrifosse G. "Immunochemical, biomolecular and biochemical characterization of bovine epithelial intestinal primocultres." *BMC Cell Biology* 6 (2005).