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Alexandria Hammons April 17, 2012

Investigation of the Functional Role of BMP Signaling in Zebrafish ENS Development

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Abstract

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The Enteric Nervous System (ENS) is the set of neurons and glia that comprises the nervous system in your intestine, allowing for complex control of gastrointestinal (GI) function. A properly constructed ENS requires sufficient proliferation followed by appropriate differentiation of these enteric precursors, which all derive from an axial population of cells called the vagal neural crest. If the precursors fail to populate the gut in sufficient numbers and fail to differentiate into the appropriate neuronal and glial subtypes, it can cause various congenital disorders, one of which is Hirschsprung's (HSCR) Disease. HSCR is a pediatric developmental disorder occurring in approximately 1:5000 live births. The genetic basis of HSCR is only partially understood. To identify new HSCR genes we utilize the zebrafish model system. Previously the Shepherd lab identified a zebrafish ENS mutant with an HSCR-like phenotype that was named *lessen* (*lsn*). In subsequent analysis of this mutant, a number of components of the Bone Morphogenic Protein (BMP) signaling pathway were differentially expressed. The BMP pathway is known to be involved in various developmental processes and has been implicated in ENS development. Given these results we aimed to determine the spatial and temporal expression pattern of the BMP ligands in the developing zebrafish intestine by *in situ* hybridizations. We then determined the effect of inhibiting BMP signaling on zebrafish ENS development. We find that treatment with a small molecule inhibitor of the BMP pathway, DMH1 at stages of ENS development when neural precursors would normally be differentiating, causes an increase in the number of enteric neurons at 120hpf. We propose a model of BMP-regulated ENS development in which BMP signaling determines different developmental choices depending on the amount of BMP signaling in the intestine at specific developmental stages. Early in zebrafish ENS development low levels of BMP signaling maintain ENS precursors in a proliferative state while levels of BMP signaling at later stages of ENS development causes precursor differentiation. Central to this model is the regulation of ID gene expression where low levels of BMP signaling enhances ID genes expression while high levels of BMP signaling represses ID gene expression.

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Introduction

Gastrointestinal tract

The gastrointestinal (GI) tract, or the alimentary canal, refers to the organ system responsible for the digestion of food. The vertebrate GI tract comprises an array of organs, which form an interconnected system from a single tubular structure. The esophagus, stomach, intestines, and colon all make up the GI tract. The gut itself consists of three segments along the anterior-posterior axis: the foregut (pharynx, esophagus, and stomach), midgut (small intestines), and hindgut (colon). Critically the radial patterning of the GI tract relies on coordinated signaling between the different germ layers. In particular, the endoderm and mesoderm engage in cross talk to generate the gut epithelium (De Santa Barbara, 2002). The endoderm forms the epithelial lining while the mesoderm forms the smooth muscles layers of the GI tract and the enteric nervous system arises from the ecotoderm.[20] The GI system must move contents through the tract by a sequence of propulsive movements called peristalsis controlled directly by the enteric nervous system (De Santa Barbara, 2005).

ENS Development

The neurons and glia of the enteric nervous system (ENS) create the intrinsic innervation of the gastrointestinal (GI) tract. The ENS comprises the largest part of the peripheral nervous system and coordinates intestinal muscle contractions, blood flow, and water, and electrolyte secretion (Burzynski, 2009). ENS neurons and glia organize themselves into ganglia, which form two interconnected plexi, the myenteric and submucosal plexi that run the length of the bowel in chains of interconnected cells which contain numerous neuronal subtypes (Heanue, 2007 and Sasselli, 2012). The myenteric plexus is primarily responsible for intestinal peristalsis while the submucosal plexus

concerns itself with regulating blood flow as well as secretion of gut hormones, exchange of fluids across the mucosal surface, and absorption (Sasselli, 2012). The two plexi are separated in that the myenteric plexus resides between the longitudinal and circular muscle layers while the submucosal plexus lies in the submucosal layer (Heanue, 2007).

In all vertebrates the majority of the ENS is derived from the vagal neural crest cells (NCCs), which enter the foregut and colonize the length of the gut in a rostro-caudal direction. In later evolving vertebrates, the sacral neural crest also makes a small contribution of cells to the ENS (Burzynski, 2009). The sacral NCC enter the hindgut after the vagal NCCs have populated it and studies in avians suggest that the sacral NCCs are not essential for the development of a functional ENS (Burzynski, 2009). Debate persists whether NCCs of the vagal and sacral regions are pre-specified. Chick and quail chimera studies involving grafting of vagal and sacral neural tube show that NCCs derived from sacral transplants still migrate to their appropriate target characteristic of their new vagal axial location. However, these cells keep some properties of their original axial position prior to grafting (Sasselli, 2012). This provides some basis for the compensatory ability of the NCCs as they populate the intestine. However, recent data from the Burns lab indicate there is sub specification for the enteric neural crest in the vagal crest (Burns, 2012)

The ENS along with intestinal smooth muscles and the interstitial cells of Cajal initiate coordinate gut peristalsis independent of innervation from the central nervous system (CNS). Critically, the development of synchronized gut motility requires the ENS (Burzynski, 2009). However, there is a time lag between the appearance of neurons within the gut and the initiation of neurally-orchestrated gut motility (Sasselli, 2012). The

ENS is composed of about 20 different types of enteric neurons based on morphology and neurotransmitter expression. This diversity of neuronal subtype is comparable to the diversity found in the CNS (Burzynski, 2009 and Sasselli, 2012). In order to create a functional ENS, ENS precursor cells must proliferate sufficiently on entering the intestine and then differentiate appropriately to generate a functional neural network (Burzynski, 2009).

Neural crest cells arise at the border between the neural and non-neural ectoderm and give rise to a multitude of neuronal and non-neuronal cell types. As mentioned previously the ENS is principally derived from a specific axial population of neural crest cells, the vagal neural crest. Vagal neural crest cells enter the foregut early in development and are subsequently termed enteric neural crest cells (ENCCs); these are the ENS precursor cells (Heanue, 2007). Proliferation of the ENCCs enables the appropriate number of neurons and glia to be generated to colonize the full length of the bowel (Heanue, 2007). This precursor proliferation is essential since only a limited number of ENCCs enters the gastrointestinal tract from the neural crest (Oden, 2008).

To generate this neural network in the gut requires the precise function of a number of different signaling pathways that act throughout development (Heanue, 2007). When factors are required early in ENS development they produce more extensive intestinal abnormalities since the group of cells they make contact with are predominantly multipotent ENS precursors (Gershon, 2004). On the same token, developmental factors required later in development typically affect smaller subsets of cells when the precursors no longer have such a broad developmental potential and thus produce smaller malformations (Gershon, 2004). However, the exact biological function, timing, and

contribution of each of these signaling pathways to the development of a functional ENS is still unknown. The requirement of these signaling pathways in the ENS is critical for growth; as such, it is no surprise that there are congenital disorders that result from the failure of proper ENS formation.

Hirschsprung's disease

Hirschsprung's (HSCR) is a pediatric developmental disorder of the gastrointestinal (GI) tract that occurs in 1 in 5000 live births (Burzynski, 2009). In the absence of proper neural crest cell colonization of the gut, the ENS fails to form correctly and results in intestinal aganglionosis, or an absence of intestinal neurons, both in the myenteric and submucosal plexi (Burzynski, 2009). HSCR is characterized by intestinal obstruction or impaired intestinal motility that results from this reduction of enteric neuron number principally in the distal portion of the GI tract (Burzynski, 2009). Apart from the lack of enteric neurons in HSCR, the aganglionic portion of the colon exhibits muscular hypertrophy as its wall thickens and the colon becomes distended (De Santa Barbara, 2002). The condition is one of the most visible disorders of the enteric nervous system (Gershon, 2004). HSCR can be familial, sporadic or manifests as a clinical symptom in various congenital syndromes (Burzynski, 2009). There are two forms of HSCR: long-segment that in some cases can result in total colonic or total intestinal aganglionosis, and short-segment, which occurs more frequently and affects the rectum and a small portion of the colon (Shepherd, 2004). It is likely that the majority of HSCR cases derive from the intricate interactions between several different susceptibility genes (Gershon, 2004). A common attribute of the genes thus far tied to the pathogenesis of HSCR is that they are required early in ENS development, explaining the large deficit

they cause in intestinal development (Gershon, 2004). Pervious genetic studies have shown that mutations in the RET coding sequence accounts for 50% of familial and 15-20% of sporadic HSCR cases.[1] While RET mutations comprise a sizeable fraction of HSCR cases, other transcription factors and signaling pathways have also been shown to underlie this disease. These additional genes have been shown to regulate specification, differentiation, and proliferation of the ENS. Other signaling pathways and ligands implicated in ENS development include: GDNF, neurturin, endothelin 3, BMP2/4, Ihh (Indian hedgehog), Shh (sonic hedgehog), NT3, and CNTF (Heanue, 2007).

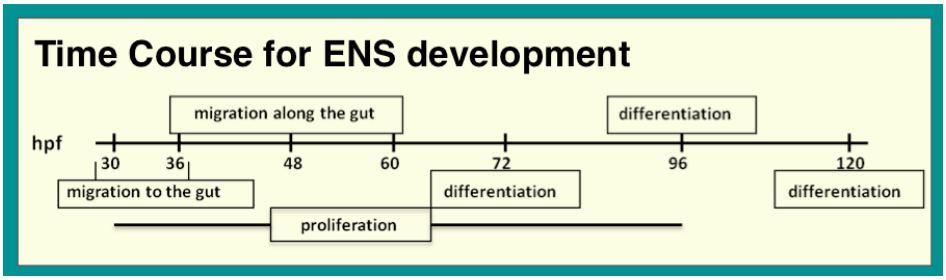

Figure 1*: Time line of ENCC and ENS development along the developing gut.*

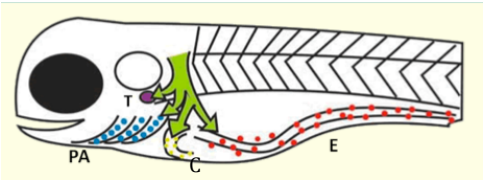

Figure 2**:** *The zebrafish ENS is derived from the vagal neural crest. Cells are contributed to the thymus (T), Phryngeal arches (PA), the cardiac outflow tract (C), and the ENS (E).*

The zebrafish model system

Zebrafish have become an important model organism in the study of vertebrate development for several key reasons. Zebrafish reach sexual maturity at approximately 3 months of age and have short generation times. A single pair of adult zebrafish can generate a clutch of between 50-100 externally fertilized eggs per week. The embryos are readily observable and are transparent.

Zebrafish also have a relatively small genome of 1.7 x 10^7 base pairs with 25 chromosomes. A large number of zebrafish mutants and GFP expressing transgenic lines

can be readily obtained from the ZIRC (Zebrafish International Resource Center) and there are a wide variety of molecular and cellular techniques that permit easy manipulation of gene expression in vivo. These characteristics make the zebrafish an excellent model organism for investigating vertebrate developmental processes (Burzynski, 2009).

ENS Development In Zebrafish

The vagal neural crest in zebrafish lies between the otic vesicle and the first somite. These cells migrate ventrally from the vagal region and give rise to the ENS as well as contributing cells to the thymus, pharyngeal arch cartilage, and the heart (Chalazonitis, 2004). From 30-36hpf, the neural crest cells migrate to the anterior end of the gut (Figure 1). Having reached the gut at 36hpf, ENCCs migrate along the length of the gut in two parallel streams on either side of the intestinal endoderm reaching its end by 60 hours. At 72hpf, the first wave of neuronal differentiation begins in the intestine. This differentiation occurs in a rostro-caudal manner. A second wave of differentiation begins at 96hpf and coincides with intestinal smooth muscle differentiation. Differentiation continues along the length of the gut through 120hpf (Chalazonitis, 2004).

Zebrafish ENS development is simpler than that in other vertebrates in that there is no sacral neural crest cell contribution to the ENS (Figure 2). Furthermore, ENCCs in zebrafish do not form multiple complex chains of migrating cells in the GI tract as seen in amniotes, but instead form two chains that run parallel to the intestine in the rostro-caudal direction. There is, however, conservation in the genes necessary for ENS development in zebrafish as compared to other vertebrates. Conserved genes required for ENS development include: *sox10, gdnf, gfrα1, phox2b*, and *sip1*. Significantly zebrafish also

have a conserved function of RET in ENS development (Burzynski, 2009). In summary, these embryological characteristics of zebrafish and the conservation of many key signaling pathways in ENS development make zebrafish an excellent model system for studying vertebrate ENS development.

***lessen* mutants**

The ENS mutant, *lessen (lsn)*, was identified during a forward genetic screen looking for zebrafish mutants that had a reduction in the number of ENS neurons at 96hpf, predominantly in the posterior region of the gut. Positional cloning identified that the *lsn* mutation resulted from a null mutation in a subunit of the mediator co-transcriptional activation complex, med24/trap100. Embryological studies in the lab show that the *lessen* ENS phenotype results from a proliferation defect of the ENS precursors once they reach the intestine. *lsn/med24* is not required for the initial steps of cranial neural crest development or vagal crest cell migration to the anterior end of the gut, but is essential for the normal proliferation of the ENS precursors at later stages of embryonic development (Pietch, 2007).

However, since this loss of MED24 expression is a comparatively common coactivator it was necessary to determine which particular signaling pathway was being affected in the *lsn* mutant. To ascertain which signaling pathways were causing the mutant phenotype the Shepherd lab undertook a microarray analysis of the gene expression profile of the mutant intestine at 48hpf compared to the wild type intestine at the same age. The microarray studies demonstrated that several BMP pathway components were differentially expressed in the mutant intestine as compared to wild type.

Critically the phenotype observed in these *lessen* mutants was not due to perturbation of the GDNF signaling pathway as expression this components of this pathway remained unchanged in mutant embryos when compared to wild type. GDNF is critical for migration of ENS precursors along the intestine (Pietch, 2007).. In addition, there was no increase in cell death of the ENS precursors in the mutants compared to wild type at either 48 or 72hpf (Pietch, 2007).

Bone Morphogenic Protein (BMP) Pathway

BMP signaling is propagated through specific serine/threonine kinase receptors where internal protein complexes shuttle the signal from the membrane to the nucleus. These complexing proteins are called Smad proteins, which were discovered as a class of transcription factors that mediated responsiveness between the cell surface and nucleus for members of the TGFβ family (Zeng, 2010). BMP receptors are of two distinct forms, type I and type II, of which there are further subtypes. BMP type I receptors include, activin receptor-like kinase 2 (ALK2), ALK3 (known as BMPR-1A), and ALK6 (BMPR-1B). BMPtypeII receptor (BRII) and Activin type II receptors Act RII and ActRIIB comprise the BMP type II receptors. When the BMP ligand binds, these heteromeric receptors phosphorylate the downstream Smad factors, primarily the R-Smad, known as the receptor activated Smad (Zeng, 2010). Before translocation into the nucleus, this R-Smad will complex with the Co-Smad. Once in the nucleus, this Smad complex can act directly as a transcription factor and will regulate BMP responsive genes by binding to DNA and also interacting with other DNA-binding proteins (Zeng, 2010). BMPs transduce signals through Smad 1/5/8 while Smad 2/3 transduce TGFβ signals. The nuclear level of Smad proteins and thus the effect it has on transcription is tightly

regulated by ubinquintin-mediated degradation along with inhibitory Smads (Smad 6/7), which interfere with activation of R-Smad. Members of the Smad ubinquintin regulatory factor 1 family of ubinquintin ligases selectively mark Smads transducing BMP signals for degredation (Zeng, 2010).

BMP Pathway in ENS Development

Bone Morphogenic Proteins (BMPs) make up a sub-group of the TGF-β secreted signaling molecules superfamily. BMPs play a role in a number of non-osteogenic developmental processes despite their name and discovery as a regulator of bone autoinduction (Chen, 2009). BMP ligands bind to their receptors causing activation of a downstream, SMAD 1/5/8 signaling cascade (Goldstein, 2005). Previous studies have shown that BMP promotes differentiation of neural crest cells to an autonomic neuronal fate at later stages of embryogenesis (Goldstein, 2005). Inhibitors of BMP signaling are expressed throughout development and help regulate this important pathway. These antagonists include, Noggin, chordin, follistatin and follistatin-related gene (FRG), ventropin, and twisted gastrulation, all of which bind BMPs and subsequently prevent their interaction with the appropriate signaling receptors (Zeng, 2010). Antagonists of BMP antagonists also help to regulate level of BMP signaling in vivo. Tolloid, for example, cleaves chordin to release BMP from its complexes with antagonists (Zeng, 2010). BMP4 and the BMP inhibitor, noggin, are both expressed during ENCC migration along the bowel wall in mice and avians. An increase in BMP signaling via inhibition of the BMP antagonist noggin has been shown to reduce the rate of ENCC migration while conversely, inhibition of BMP signaling by antibodies or by over expression of noggin causes an increase in ENCC migration in gut explants (Fu, 2006). Increases in BMP

signaling have also been shown to promote ganglion cell aggregation and neurite fasciculation (Fu, 2006). Additional mouse embryological studies that investigated the expression of BMP and its receptors, and examined the affect of BMP antagonists on ENS development using transgenic mice suggested that BMP signaling regulates ENS neuron numbers as well as helps determine neuronal sublineages (Chalazonitis, 2004). A role for BMP is suggested to determine the diversity of enteric neuron phenotypes through BMP's regulation of when cells exit the cell cycle (Sasselli, 2012). The BMP antagonist Noggin has been utilized to show how BMP concentrations can impact when a cell is prompted to leave the cell cycle. When Noggin was used to inhibit BMP signaling, it was reported that the proportion of cells that exit the cell cycle early in development was increased while the numbers of cell types known to exit cell cycle later in development was reduced (Sasselli, 2012). This suggests that in response to changing concentrations of BMP, cells may be stimulated to leave the cell cycle at critical developmental time points (Sasselli, 2012).

Other studies also implicate the temporal requirement of BMPs 2 and -4 during gut morphogenesis and also in the adult organism. BMPs are expressed after the conclusion of ENS development. Gershon and Ratcliffe demonstrated that BMP is expressed by the mucosal connective tissue in the mature bowel. At this stage BMP is believed to help form intestinal crypts and act on colonic epithelial cells (Gershon, 2004). Together these data suggests that BMP signaling plays multiple roles in ENS development and function. These activities include regulating in ENCC migration, proliferation and differentiation along the GI tract; often acting in a concentration-dependent manner (Gershon, 2004). However, the precise temporal and spatial

requirement of BMP signaling in the developing intestine of zebrafish and how this affects ENS and GI development remains unknown. Previous avian and murine studies suggest that there is a concentration-dependent effect of BMP signaling on ENS development. ENCC development is dependent on their local microenvironment in the intestine and this regulates cell proliferation and differentiation (Gershon, 2004) Furthermore, studies imply that small changes in BMP signaling have widespread effects on ENS and GI development and warrant further investigation (De Santa Barbara, 2005). The drug DMH1 is a small molecule inhibitor of the BMP signaling pathway. DMH1 is an analogue of the BMP antagonist dorsomorphin (DM). DMH1 is a more selective inhibitor for the BMP type I receptor than DM. Both DMH1 and DM work by inhibiting ligand dependent receptor phosphroylation and thus block the subsequent downstream signaling cascade (Hao, 2009).

Based on the known role of BMP in ENS development in other species and our data gathered from the *lessen* mutant microarrays, we decided to further investigate the role of BMP signaling in zebrafish ENS development. Using DMH1 we perturbed BMP signaling in vertebrate embryos at different time points associated with proliferation and differentiation of ENCCs. We find that inhibition of BMP signaling during early stages of ENS development causes an increase in enteric neuron number. To explain this result we propose a model for BMP signaling during ENS development where different developmental programs are activated dependent on the level of BMP signaling in the ENS. During early ENS development low levels of BMP signaling promotes precursor proliferation due to enhanced expression of inhibitor of differentiation (ID) genes. At later stages of ENS development higher levels of BMP signaling causes ENCC

differentiation due to repression of ID gene expression. Results from this study are of potential clinical significance as they will clarify the role of BMP signaling in ENS development and in turn this knowledge will have a direct impact on new methods being developed to treat HSCR using ENCC stem cells.

BMPs provide critical signals for determining myriad processes in development; most all terminally differentiated or specialized cells will encounter BMP signaling. It is likely that critical bone morphogenic protein signals will be encountered in at least one or more stages as they proceed through specification from *multipotent* progenitors (Hong, 2009). The likelihood of encountering BMP signaling is further enhanced by the more than 20 structurally diverse BMP ligands that have been identified (Hong, 2009).

Materials and Methods:

Zebrafish maintenance and breeding

Zebrafish embryos were generated by crossing adult zebrafish pairs. Embryos were collected throughout the day after the pair was allowed to begin to mate. Collected embryos were sorted, placed in petri dishes containing embryo media and allowed to develop in an incubator at 28.5°C, in preparation for the drug treatments (Westerfield, 1993).

Temporal Effect of BMP Signaling on ENS Development

To investigate the temporal effect of BMP signaling on ENS development, embryos were treated with 0.5μM DMH1 as well as a control of DMSO. The drug was applied to staged embryos for three shorter time intervals: 24-72hpf, 48-72hpf, and 72-120hpf; as well one longer treatment from 24-120hpf. Wild type embryos were used for this study. Embryos were fixed at 120hpf using a 4% paraformaldehyde/1X fix buffer for 1-2 hours at room temperature or overnight at 4^{0}C for 8-16 hours and processed for either immunocytochemistry or whole-mount in situ techniques described below. The purpose of this was to assess neuronal differentiation and intestinal muscle development in experimental and control embryos. This experiment was done in triplicate.

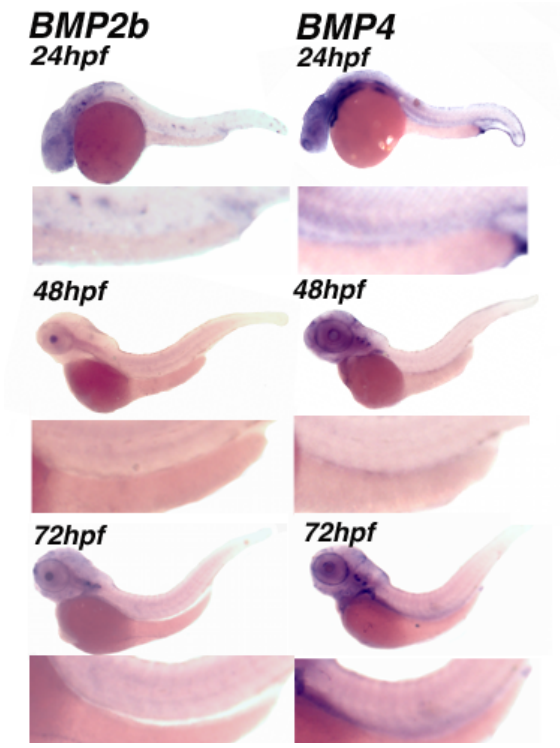

Fig. 3 *Whole mount In situ hybridization showing expression of the mRNA transcripts for BMP2b and BMP4. Expression was determined for the developmental timepoints critical for ENS development in zebrafish. Of the two transcripts, BMP4 is most strongly expressed in*

Immunocytochemistry

Embryos were processed for immunocytochemistry as described by Raible and Kruse (2000). Enteric neurons were stained using an anti-Hu mAB 16A11 (Molecular Probes) antibody, that labels differentiated neurons. An Alexa Flour 563 conjugated goat anti-rabbit IgG antibody was used as a secondary antibody.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out according to the methods previously described by Thisse et al. (1993). Digoxigenin-labelled riboprobes for *bmp2b, bmp4, bmp receptor 1 and bmp receptor 2* as well as probes for intestinal smooth muscle genes myosin heavy chain 1 and smooth muscle actin were synthesized from templates linearized with *BamH1* and transcribed with SP6 and T7 promoters. The expression of the BMP pathway genes was used to establish the temporal and spatial expression pattern of these genes in the intestine in relation to the developing ENS. The muscle specific probes, *myh11* (staining for specification of muscle tissue) and *sma* (staining for differentiated smooth muscle tissue) were used to determine intestinal smooth muscle development in DMH1 treated and DMSO control embryos. The pattern of expression of these genes was visualized using NBT/BCIP coloration reactions (Thisse, 1993).

Statistical Analysis

Using Microsoft Excel, enteric neurons were counted and analyzed using the "Student's T-Test," as a way to compare the means of the control embryos versus the drug treated embryos using the null hypothesis that the means of measurement variable are equal for each of the two groups. The test assumes the observations in each sample are normally distributed and the variances within each group are equal (Burton, 2002).

Results and Discussion:

Expression of BMP signaling components

Patterns of BMP2b and BMP4 expression were determined in the developing

zebrafish embryo and found to be consistent with data shown from previous studies indicating BMP expression in the gut is consistent with its role in ENS development (Goldstein, 2005). Goldstein et al. showed that in avian BMP4 is expressed along the length of the hindgut at all stages of development that were examined (E7-E14); this coincides with the arrival of the enteric precursors in the avian intestine (Goldstein, 2005). Our findings show a similar pattern of expression for BMP4 in the zebrafish intestinal tract (Figure 3). Specifically, expression of the BMP mRNA showed a strong expression in the zebrafish intestine at 72hpf, consistent with the initiation of differentiation of neuronal precursors in the gut. BMP2b showed less expression in the gut at all developmental stages examined. Expression of the BMP2b transcript was strongest in the head of the developing embryo. This expression in the head and less in the intestine is consistent with data reported by Thisse et al. (2001) of BMP2b expression in the zebrafish embryo at 19-22hpf, just prior to the times examined in this study.

A spatiotemporal pattern of expression for these BMP transcripts was needed to provide a basis for investigating the effect of perturbing BMP signaling on ENS and intestinal development. Shepherd lab is in the process of determining expression pattern of the receptors for BMP2 and BMP4 in zebrafish to gain a more comprehensive understanding of the overall expression of BMP and its receptors. However, expression of the BMP ligands and their receptors is not always indicative of where the BMP signaling cascade is initiated especially given the large amount of regulatory molecules involved in this pathway (Goldstein, 2005). Antibodies detecting phosphorylated Smad 1,5,8 (P-Smad) are typically used to determine the endogenous spatiotemporal pattern of BMP signaling. In avian, P-Smad expression is detected at E8 in the submucosal plexus

and the avian specific nerve of Remak (a large-ganglionated chain running along the mesentery adjacent mid- and hindgut) (Goldstein, 2005). Given our understanding of the BMP signaling pathway, using P-Smad to determine where during ENS development in zebrafish this pathway is active would be critical in understanding our proposed model of BMP-dependent ENS development.

Dose Response Curve

In order to see how the BMP pathways is involved in ENS development in zebrafish we took advantage of the small molecule inhibitors Dorsomorphin (DM) and its analogue DMH1. To use these drugs it was necessary to establish an effective concentration at which BMP signaling is inhibited. To do this we used prior knowledge of how BMP signaling is implicated in dorsoventral patterning of vertebrate embryo. Previous studies have shown that inhibition of this signaling pathway results in embryo dorsalization at early stages of development. The most commonly used small molecule inhibitor of the BMP signaling pathway, DM was first identified in a phenotypic screen by its capability to reproduce the dorso-ventral patterning defects characteristic to BMP pathway mutants. The extent to which DM cause dorsalization of the embryo varied as a function of dosage and timing (Hong, 2009). One caveat to using s mall molecule inhibitors is that there exists nonspecific perturbations in pathways unrelated to the pathway of interest. These off-target effects can lead to nonspecific events including rapid death or developmental arrest that prohibit further investigation. As this is a common difficulty encountered when developing screens, further studies have been undertaken to identify new small molecule inhibitors that are less toxic and which are more selective (Hong, 2009). DM is known to have significant off-target effects, likely a

result of its structural homology to compounds involved in the VEGF pathway and several other tyrosine kinase receptors. DM also has a limited metabolic stability in some models and undergoes microsomal degredation (Hong, 2009). Several groups have developed several DM analogues to overcome these problems by modifying functional groups and the heterocyclic core of DM (Hao, 2009). As a result, the selective BMP inhibitor, DMH1 was developed which more specifically targets the BMP type I receptor than DM, and also shows a reduced KDR (VEGF receptor type 1) activity (Hao, 2009). Both DMH1 and DM work by inhibiting ligand dependent receptor phosphroylation and thus block the subsequent downstream signaling cascade (Hao, 2009). DMH1 also selectively inhibited the BMP-induced Smad 1/5/8 activation without initiating the MAPK signaling pathway. The authors who first identified DMH1 suggested that the modification at the 3-position of the pyrazolo[1,5-a]pyrimidin-3-yl in the DM backbone potentially causes the increased BMP receptor selectivity of DMH1 (Hao, 2009).

In order to determine an effective concentration of DMH1 at which BMP signaling is blocked, we generated a dose curve for DMH1 and DM. DMH1 was applied from 0-24hpf at concentrations of .5μM, 1.0μM, 1.5μM, 2.0μM, 5.0μM, and 10.0μM. A second group of embryos were subjected to DM drug treatments at the same concentrations (Figure 4). At all concentrations tested, DMH1 treated embryos displayed a more dorsalized phenotype at earlier stages in development when compared to the DM treatment embryos. The tail of the zebrafish was lost and other ventral structures were not clearly visible at a concentration of .5μM DMH1. DMH1 appears to be about four times as potent as DM. A dosage of .5μM DMH1 was selected for our BMP signaling studies of ENS development since embryos treated with this concentration from 1-24hpf resulted

in a severely dorsalized phenotype without lethality.

An important downstream gene target of the BMP signaling pathway are the inhibitor of differentiation (ID) genes (Du, 2010). The ID genes are believed to be one of the genes whose transcription is regulated by the Smad proteins after this signaling cascade is initiated by the binding of BMP ligands. Specifically, Smad4 is known to interact with the inhibitors of DNA-binding proteins (Ids) which then regulates ID gene expression in various cell types. ID proteins are suggested to bind bHLH transcription factors thus preventing them from binding DNA and transcribing genes. These ID proteins are implicated in many different processes within the cell including differentiation and cell cycle progression (Du, 2010). Results by Du et al. show that BMP4 and BMPRIb are co-localized with Id proteins in developing mouse retinal ganglion cells. The group also demonstrated that noggin-mediated inhibition of BMP prevented Smad phosphorylation and the upregulation of Id proteins (Du, 2010). Unpublished data from the Fritz lab indicates there are SMAD and SIP1 repressor binding sites are located within the regulatory regions of the ID genes. Furthermore, microarray analysis revealed that ID2 and ID3 show significantly lower levels of expression in the intestines of *lsn* mutants as compared to wild types embryos (unpublished data).

ENS Development in DMH1 Treated Embryos

Enteric precursors continue to proliferate and differentiate into various subtypes of neurons as they migrate along the intestine. Precursors differentiate first anteriorly and the wave of neuronal differentiation continues in an anterior to posterior manner (Oden, 2008). Ensuring that the precursor pool remains sufficiently large is a critical function of

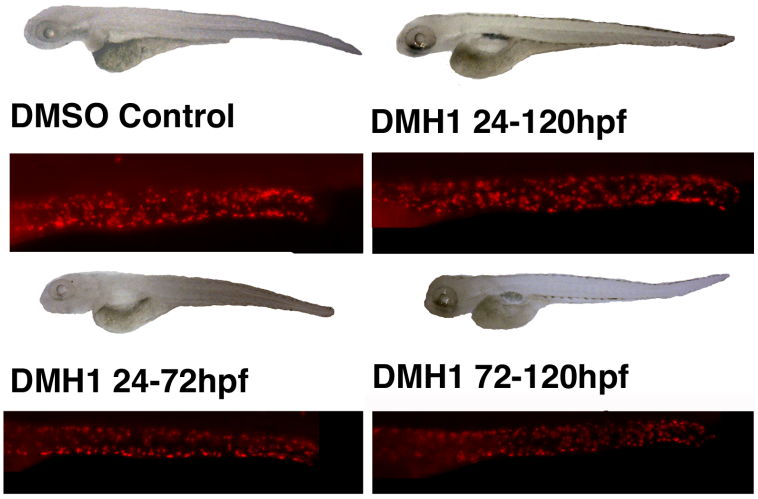

Figure 5: *Embryos treated with DMH1 from 24-120hpf exhibited on average more neurons than in the DMSO control. DMH1 treatment from 24-72hpf showed no increase in intestinal neurons compared to the control. In contrast, embryos treated with DMH1 from 72-120hpf, had in total more neurons than were present in the control.*

the invading cells at the beginning of the wave front (Oden, 2008). The importance of proliferation is further demonstrated in studies that have highlighted the influence of the population size of the pre-enteric NCC. It has been suggested there exists a minimal number of NCC necessary for colonization of the gut by neural precursors (Sasselli, 2012). Certain mouse mutant lines showed a decrease in the amount of neurons that populated the length of the intestine or even aganglionosis of the terminal gut when there was a reduction of enteric progenitors. It has also been suggested that the speed the wave front colonizes the intestine is influenced by the initial number of ENS precursors; in embryos with smaller precursor pools, the speed of migration of ENS precursors through the gut was reduced (Saselli, 2012).

Previous studies by Chalazonitis et al. (2004) have shown that BMP signaling limits the total neuron number in the ENS. This manifests as a preference for later born neuronal phenotypes over earlier ones; this ultimately changes the overall composition of the ENS and affects the glia to neuron ratio. It is suggested that BMPs modulate the pool

of neurons fated for both the neuronal and glial phenotypes. Furthermore, increased BMP signaling later in development, E12 in mice, is thought to enhance glial development at the cost of the number of neurons. In contrast, a decrease in BMP signaling at this stage is suggested to do the opposite and promote neurogenesis over gliogenesis. BMP acts on both of these processes in a time-dependent manner (Chalazonitis, 2011).

ENCCs at the migratory front also show signs of containing a higher number of mitotic cells than those more anterior; suggesting that precursors in the posterior region have a shorter cell cycle (Oden, 2008). The commitment of neuronal precursors is linked to their continuance to remain in an undifferentiated state and therefore the modulation of transcriptional regulators which inhibit neuronal differentiation must be down regulated in order for ENCC to undergo appropriate subtype specification (Saselli, 2012). It is this change in the number of cell cycles that progenitors undergo that may explain why a change in BMP signaling wields contrasting effects on neurons born early and those which are born later in development. An increase in BMP signaling is shown to increase the amount of late born neurons and decrease those born early while a decrease in BMP signaling exerts the opposite effect. BMP is believed to be critical in the differentiation of later born neuronal populations, explaining how it can alter the size of this late born pool (Chalazonitis, 2011).

Olden et al. have made the suggestion ENCCs most posterior are those which will populate the latter portions of the gut as opposed to producing precursors that would move into the remaining un-innervated regions of the gut (Oden, 2008). They believe that the pattern of differentiation among ENCCs in the posterior region of the gut suggests that at the end of ENCC migration, a small group of enteric precursors may remain in a more proliferative state. These ENCCs that persist as precursors are those that will produce the progeny that will colonize the intestine's more posterior regions (Oden, 2008).

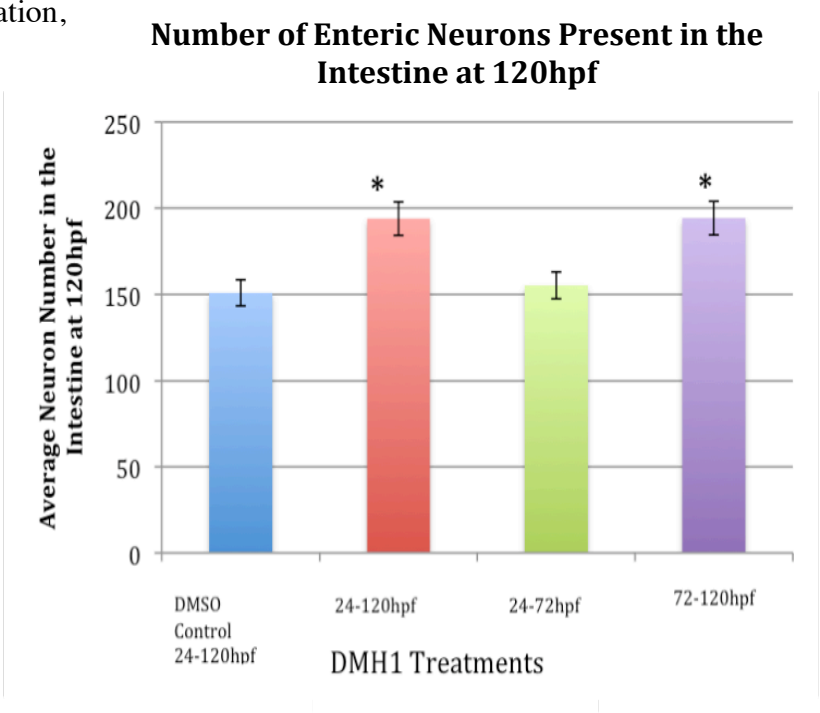

Fig. 6: *The average number of enteric neurons in the intestine present at 120hpf when treated with DMSO (control) and DMH1 at time intervals known to be important for proliferation and differentiation in of ENCCs in zebrafish ENS development.*

Embryos treated with DMH1 from 24-120hpf exhibited, on average, more neurons than were observed in the control embryos (Figure 5 and Figure 6). DMH1 treatment from 24-72hpf and treatment from 48-72hpf (data not shown) resulted in no significant increase in neuron number as compared to controls (on average 155.26 and 151.03 neurons, respectively, in the intestine compared to the 150.9 intestinal neurons in the DMSO control). Embryos treated with DMH1 later in development from 72hpf-120hpf showed an increase in the number of intestinal neurons over the control DMSO, 194.2 neurons on average in DMH1 treatment as compared to

150.9 in controls. These results suggest that BMP plays a critical role in regulating ENCC choice between proliferating and differentiating during ENS development. To explain this outcome we propose a model for BMP signaling during ENS development in which BMP concentrations regulate this decision.

It is postulated there may be an optimal level of BMP allowing for differentiation to occur, fitting with our model of a threshold of BMP concentration at which different developmental pathways are initiated at low or high concentrations. It has been shown the cell's survivability may be compromised at significantly increased level of BMP or prolonged exposure to BMP ligands. Chalazonitis et al. (2004) showed that in cultures of ENCC treated with high concentration of BMP2 or BMP4 an increasing proportion of cells underwent apoptosis after prolonged treatment (six days) as opposed to the maximal neuronal differentiation they found in response to high concentrations of these two BMPs for a short exposure of only two days. They concluded that BMP2 and BMP4 induce optimal neuronal differentiation of ENCC precursors in a concentration and time dependent manner; time referring to both the length of treatment as well as the specific time of development at which the BMP was administered to the cultured cells (Chalazonitis, 2011). Additionally, data from the CNS showed that increased amounts of BMP2 and BMP4 promoted apoptosis and inhibited cell proliferation when treated cells were at an earlier developmental age. In contrast, when CNS progenitor cells were treated at later stages of development, BMP2 and BMP4 promoted neuronal differentiation, in concordance with our findings that BMPs are increased at later stages of development and involved in differentiation to a neuronal fate (Chalazonitis, 2011).

Our data on DMH1 treated embryos as well as work done by other labs supports a model for BMP-dependent ENS development in which ENCCs respond to a threshold concentration of BMP in order to make the developmental choice to proliferate or differentiate. We propose a BMP concentration curve (Figure 7) where ENCCs initiate different developmental pathways in response to changing concentrations of BMP signaling. At early stages of ENS development, low levels of BMP signaling lead to expression of ID genes, which promotes ENCC proliferation. We suggest that this low level of BMP signaling promotes maintenance of a proiliferative state since the ID genes are inhibiting the ENCCs from differentiating. We propose that there exists a threshold concentration of BMP at which the precursor neurons will normally be stimulated to differentiate. At later stages in ENS development higher levels of BMP signal lead to repression of the ID genes and that in turn causes ENCC to differentiate. This is demonstrated in the embryos treated with DMH1 from 24-120hpsf and 72-120hpf. In both cases, the small molecule inhibitor represses BMP signaling,

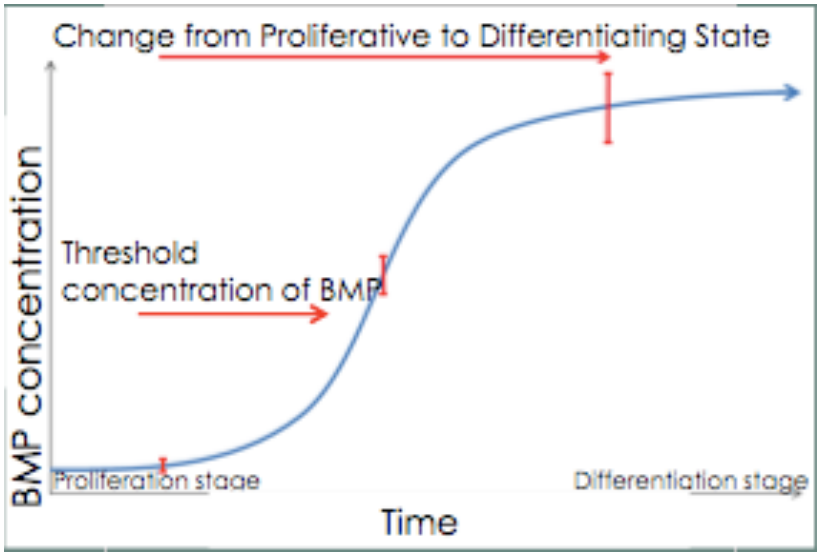

Fig. 7: *Model demonstrating that at different developmental time points different developmental pathways are initiated, depending on the concentration of BMP signaling. At early stages of development, BMP is in low concentrations and thus neuronal precursors are maintained in a proliferative stage. During later development where BMP is in higher concentrations, these ENCCs are stimulated to differentiate into neurons.*

resulting in low levels of P-Smad and thus expression of the ID genes. ID gene expression maintains their proliferative state during a developmental time point at which these neuronal precursors would normally begin to differentiate. Essentially, this BMP concentration curve will be shifted to the right when BMP signaling is inhibited by DMH1 such that the threshold is not reached until a later time. We observe a larger number of differentiated neurons present in the intestine at 120hpf because once the threshold concentration of BMP is reached there is a larger pool of precursor neurons available to differentiate.

We observed no changes in the number of enteric neurons with DMH1 treated embryos from 24-72hpf or 48-72hpf because we have not abnormally increased the length of time ENCC are maintained in a proliferative state. If DMH1 is administered early or in the middle stages of ENS development BMP signaling is kept at approximately the same levels as would be normally experienced during this proliferative phase of ENS development. In these treatments, the DMH1 is not delivered long enough to maintain the proliferative state to result in a larger precursor pool that will subsequently differentiate into neurons. Removal of the drug thus occurs at a stage when the gut is only beginning to increase in concentration of BMP signaling and the cells can then respond to the normal levels of BMP signaling later in development.

Effect of DMH1 treatment on intestinal smooth muscle development

Periods, or waves, of differentiation of enteric neurons closely corresponds to development of circular and longitudinal smooth muscle, with an actual increase in neuronal differentiation during muscle differentiation (Oden, 2008). Development of the

smooth muscle tissue, which the enteric neurons will innervate, is important in establishing a functional gut. Differentiation of circular smooth muscle begins on the second day of embryogenesis as indicated by its expression of smooth muscle myosin heavy chain (*smmhc*), one of the first markers of smooth muscle development (Oden, 2008). This period, which occurs between 63-69hpf, sees a 2.5 fold increase in neuronal density and is the time at which axons will begin to project (Oden, 2008). The ENS experiences further periods of rapid increases in differentiation, one of which is during the first phase of longitudinal differentiation between 82-89hpf and another during smooth muscle development between 93-98hpf (Oden, 2008). It's hypothesized that an increase in neuronal differentiation coincides with muscle development as the forming muscle may be able to provide the scaffolding for the nascent neurons to initiate their connectivity and path finding. This is presumed to continue during later longitudinal muscle differentiation giving the differentiating ENCCs the framework for creating a functional network of neurons (Oden, 2008). In zebrafish, differentiation of circular smooth muscle coincides with axon projection of neurons that move around the circumference of the intestine (Oden, 2008).

Critically for this investigation, BMP2 and BMP4 have been shown to promote differentiation of the circular and longitudinal smooth muscles.[23] Data in chick also shows that inappropriate activation and inhibition of endogenous BMP activity can result in an array of defects in the mesenchyme-smooth muscle, epithelium, and enteric system of the gut (De Santa Barbara, 2005). In mice, treatment of non-ENCC cells that induce smooth muscle actin (SMA^+) with BMP2 and BMP4 increases their number of these cells by more than 50% at E12. Yet by E14, numbers of SMA^+ cells are reduced and treatment

with either BMP2 or -4 does not increase population size (Chalazonitis, 2011). Both of these findings are consistent with chick where studies show that BMP4 enhances smooth muscle development (Chalazonitis, 2011). Additional support for the role BMP in smooth muscle development comes from Noggin studies. In the fetal gut of E18 transgenic mice over-expressing Noggin developed a circular muscle layer but not a layer of longitudinal muscle. Overall these data suggest that BMP has a stimulatory effect on the differentiation of smooth muscle precursors during fetal development, but an inhibitory effect later in development (Chalazonitis, 2011). Critically, it is believed that since BMP signaling is shown to regulate smooth muscle differentiation and axonal elongation and neuronal fasciculation, it can coordinate proper functional exchanges between the presynaptic neurons and the smooth muscle that they innervate (Heanue, 2007).

As BMP has been implicated in regulating differentiation of smooth muscle, we examined the effect of DMH1 treatment on smooth muscle development through. We treated zebrafish experimental and control embryos with DMH1 for the same developmental time points as that undertaken in our analysis of the effect of DMH1 treatment on total ENS neuronal number (Figure 8). We analyzed intestinal smooth muscle development by performing *in situs* using a probe for *sma*. All staining was done at 120hpf as well as a control DMSO fish treated from 24-120hpf. In each treatment of fish and in the control, there were no observable defects in the morphology of the smooth muscle. The differentiation of precursors into smooth muscle cells was not affected by inhibition of BMP signaling. Additionally, *in situs* were performed on DMH1 treated embryos using a probe for mhy11 a probe that labels muscle precursors cells (data not shown). In comparison to the control embryos, fish in which BMP signaling was

inhibited from 24-72hpf, 24-120hpf, as well as 72-120hpf resulted in no apparent change in expression of either of these smooth muscle markers. It should be noted this was a qualitative study using in situ hybridization assess smooth muscle differentiation. Further studies using antibodies to smooth muscle actin as well as histological examination of the intestinal development would be required to definitively determine if there is no effect of DMH1 treatment on intestinal smooth muscle development. As previous data has shown that different concentrations of BMP signaling can effects smooth muscle differentiation, perhaps the absence of a distinct difference in the differentiation of this tissue in treated embryos compared to controls may suggest there are either different BMP concentrations that allow for smooth muscle differentiation and differentiation of ENCCs. Another possibility may implicate the role of other proteins or trophic factors that act with BMP to establish smooth muscle from its precursors and that are sufficient to permit normal intestinal smooth muscle

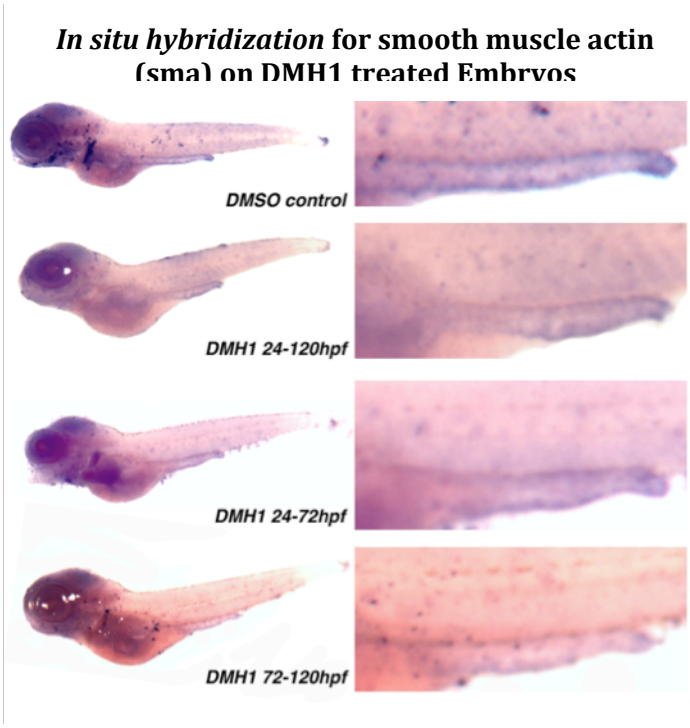

Fig. 8: *In situ Hybridization showing the effect of DMH1 treatment and consequent BMP signal inhibition on the development of the smooth muscle of the intestine. This is visualized using a probe for smooth muscle actin. Treatment with DMH1 does not appear to effect the differentiation of the smooth muscle tissue in the gut as each of the drug treated embryos had similar gut morphology and pattern of differentiation to the controls.*

differentiation in DMH1 treated embryos. These factors could be different for smooth muscle differentiation as compared to ENCCs differentiation.

Conclusions:

The enteric nervous system (ENS) is derived from precursor cells that predominantly arise from the vagal neural crest in all vertebrate species. The progression of undifferentiated enteric neural crest cells (ENCCs) to differentiated enteric neurons and glia involves many cellular signals and pathways, one of which is the bone morphogenic protein (BMP) pathway. We believe BMP regulates genes involved in controlling neuronal differentiation. Hirschprung's Disease (HSCR) is a condition that results from a failure of normal neural crest cell colonization of the intestine. This failure can result from either: a failure of the ENCC precursors to proliferate sufficiently in the intestine, or if these precursors fail to differentiate appropriately. To examine BMPs regulatory role in ENS precursor proliferation and differentiation we established a dose response curve for the small-molecule inhibitor DMH1. Subsequently we defined the effects of perturbing the BMP signaling pathway on ENS development. We found that DMH1 causes an increase in the number of enteric neurons at 120hpf if zebrafish embryos were treated with DMH1 from 24-120hpf or from 72-120hpf. No change in enteric neuron number was observed in embryos treated with DMH1 from 24-72hpf or 48-72hpf. We propose a model in which lower levels of BMP signaling initiates different developmental pathways than higher levels BMP signaling. Early in development the low amount of BMP signaling allows for expression of the inhibitor of differentiation (ID) genes, which promotes ENCCs to continue to proliferate. However, BMP signaling

increases later in development and a threshold concentration is reached which causes the ENCC to differentiate. It is at this threshold concentration of BMP signaling that ID gene expression is now repressed thus causing ENCC differentiation.

Future directions include repeating and confirming our preliminary observation. Furthermore we propose to further examine the effect of the DMH1 treatment on intestinal smooth muscle development. We propose to determine whether treatment with DMH1 affects the number of ENCC precursors by utilizing Phox2b, GFP transgenic line of zebrafish. We will repeat DMH1 treatment with the phox2b line and then stain embryos with an anti-HuC antibody as well as an anti-GFP antibody at 120hpf. Using this approach we will be able to determine the ratio of ENCC number to differentiated enteric neuron number. Although differentiation of enteric neurons during ENS development is relatively constant as they form specific subpopulations of neurons at different developmental ages, there is a difference in the pattern of differentiation among precursors at the opposing ends of the migratory wave front. Enteric neurons differentiate in an anterior to posterior wave and do not differentiate as group en masse at the same time along the length of the intestine. This pattern of enteric neural differentiation is thought to be a result of the difference in anterior and posterior neuronal density established towards the later stages of intestinal development. Furthermore, anterior region also has a lower rate of proliferation than those more posterior (Oden, 2008). An analysis of the proportion of differentiated and undifferentiated neurons using the phox2b transgenic line would help determine if BMP concentrations are critical in allowing differentiation ENCC or ending ENCC proliferation. This property of local specification

may explain how BMP could be involved in the differentiation of neurons in different regions of the wave front.

Other trophic factors are also known to be involved in regulating in vivo levels of BMP and it is thought that BMP helps strike a balance through interaction with glial derived neurotrophic factor (GDNF). In treatments of cells where BMP exposure is short and the concentration is kept low, GDNF imparts survival effects that enhance neurogenesis in ENCCs (Chalazonitis, 2011). Song et al. (1998) demonstrated this cooperation of BMP2 with other neurotrophic factors in immortalized sympatho-adrenal progenitor cells (MAH) (Chalazonitis, 2011). The composition of what factors may communicate with BMP in this network of interacting factors currently remains unknown. Future work could be undertaken in vitro to establish candidate factors that interact with BMP. This may lead to a better understanding of how BMP can act in so many different developmental processes.

In this investigation we examined the expression pattern of BMP2b and BMP4 and propose to examine it's receptors BMPR1 and BMPR2, however to fully understand the interactions of the BMP ligands with their receptors it would be advantageous to analyze P-Smad immunofluorescence. As a downstream component of the BMP signaling pathway, levels of P-Smad indicate where BMP signaling is occurring. Currently the probes used here can only tell us if our components are expressed at the right time and in the correct location to be involved in ENS development.

We also propose to attempt to rescue the *lessen* mutant ENS phenotype by perturbing BMP signaling during the critical stages of ENS development. Using wild type embryos we would inject them with med24MO. Starting around 36hpf we would

then treat control and experimental embryos with different concentrations of DMH1 then determine the effect on enteric neuron number in morphant and wild type embryos 120hpf.

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