Endoderm-derived Sonic hedgehog and mesoderm Hand2 expression are required for enteric nervous system development in zebrafish

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ABSTRACT

The zebrafish enteric nervous system (ENS), like those of all other vertebrate species, is principally derived from the vagal neural crest cells (NCC). The developmental controls that govern the migration, proliferation and patterning of the ENS precursors are not well understood. We have investigated the roles of endoderm and Sonic hedgehog (SHH) in the development of the ENS. We show that endoderm is required for the migration of ENS NCC from the vagal region to the anterior end of the intestine. We show that the expression of shh and its receptor ptc-1 correlate with the development of the ENS and demonstrate that hedgehog (HH) signaling is required in two phases, a pre-enteric and an enteric phase, for normal ENS development. We show that HH signaling regulates the proliferation of vagal NCC and ENS precursors in vivo. We also show the zebrafish hand2 is required for the normal development of the intestinal smooth muscle and the ENS. Furthermore we show that endoderm and HH signaling, but not hand2, regulate gdnf expression in the intestine, highlighting a central role of endoderm and SHH in patterning the intestine and the ENS.

Introduction

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system and is derived from the neural crest (Furness, 2006; Le Douarin and Kalcheim, 1999). In all vertebrate species studied the majority of the ENS is derived from vagal neural crest cells (NCC) (Burns, 2005; Heanue and Pachnis, 2007; Newgreen and Young, 2002b). ENS NCC migrate from the vagal region and then associate with the rostral end of the intestine. Subsequently these ENS precursors migrate rostro-caudally along the length of the intestine, continuing to proliferate while they migrate, then differentiate to form a wide variety of neuronal subtypes (Furness, 2006). The migration, proliferation and differentiation of the ENS precursors in the developing gut is dependent on reciprocal signaling interactions between the precursors, the gut mesenchyme, and the gut endoderm. Molecules in the environment as well as lineage restrictions within the ENS precursors determine the number and specific cell fates acquired by these precursors. Failure of ENS precursors to colonize the complete length of the gut results in the absence of enteric ganglia along varying lengths of the colon (colonic aganglionosis) (Heanue and Pachnis, 2007; Newgreen and Young, 2002a,b). This is the most common cause of congenital intestinal obstruction in humans and is clinically referred to as Hirschsprung's disease (HSCR) (Brooks et al., 2005; Gershon and Ratcliffe, 2004).

The molecular mechanisms that control the specification, proliferation and differentiation of the enteric neural crest have been studied extensively in vivo and in vitro. A number of transcription factors have been implicated in ENS development, including Mash1 (Guillemot et al., 1993), Phox2b (Pattyn et al., 1999), SOX10 (Herbath et al., 1998; Kapur, 1999; Pattyn et al., 1999; Southard Smith et al., 1998), Hox11L1 (Tkx2) (Hatano et al., 1997; Shirasawa et al., 1997), Hoxb5 (Kuratani and Wall, 1992; Pitera et al., 1999), HAND2 (Cserjesi et al., 1995; D’Autreaux et al., 2007; Hendershot et al., 2007; Howard et al., 1999; Srivastava et al., 1995; Wu and Howard, 2002) and AP-2alpha (Barralillo-Gimeno et al., 2004; Knight et al., 2003; O’Brien et al., 2004). Several secreted signaling molecules and their associated receptors have also been identified that control directly and indirectly the morphogenesis of the ENS. These include GDNF (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Schuchardt et al., 1994), Neurturin (Heuckeroth et al., 1999, 1998; Rossi et al., 1999), Endothelin-3 (Baynash et al., 1994; Hosoda et al., 1994; Yanagisawa et al., 1998), BMP 2/4 (Chalazonitis et al., 2004; Goldstein et al., 2005; Sukegawa et al., 2000; Wu and Howard, 2002), NT-3 (Chalazonitis et al., 2001, 1994), CNTF (Chalazonitis et al., 1998), and Indian hedgehog (Ramalho-Santos et al., 2000). Perturbation of the function of most of these transcription factors and signaling molecules/receptors leads to defects in the ENS. Furthermore, mutations in...
some of these genes have been identified in patients affected with HSCR (Amiel and Lyonnet, 2001; Brooks et al., 2005; Puri et al., 1998). Recently we have shown that endoderm-derived cues are essential for normal ENS development in zebrafish (Pietsch et al., 2006). To further investigate the role of endoderm in zebrafish ENS development and identify specific endoderm dependent signaling pathways involved in this developmental process, we have focused on the in vivo function of the hedgehog (HH) signaling pathway, and specifically Sonic hedgehog (SHH), in zebrafish ENS development.

SHH is a member of the Hedgehog family of signaling molecules that was identified by its homology to the Drosophila segment polarity gene hedgehog. SHH controls the differentiation and proliferation of numerous cell types in a variety of tissues (Ingham and McMahon, 2001; McMahon et al., 2003). In all vertebrates examined SHH is expressed along the rostro-caudal extent of the gut endoderm (Bitgood and McMahon, 1995; Ecchelard et al., 1993; Krauss et al., 1993; Marigo et al., 1995; Roberts et al., 1995; Stolow and Shi, 1995). Subsequent studies have demonstrated that SHH signaling is required for normal patterning of the gut mesenchyme and the ENS plexuses in mouse and avian systems (Fu et al., 2004; Ramalho-Santos et al., 2000; Sukegawa et al., 2000). More recently it has been shown in vitro that SHH regulates ENS NCC proliferation (Fu et al., 2004). This is significant as proliferation has been recently shown to be a major mechanism driving the invasion of ENS NCC along the gut (Simpson et al., 2007). Furthermore, Fu and colleagues’ study suggested that SHH modulates ENS NCC’s chemotactic responsiveness to GDNF. GDNF has been previously shown to act as a chemoattractant for ENS precursors (Marusich et al., 1994)
or by Immunohistochemistry (Natarajan et al., 2002; Young et al., 2001).  

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In this work we have further investigated the role of endoderm in zebrafish ENS development. We show that endoderm-derived factors are required not only for the migration of ENS precursors along the intestine, but also for the initial migration of NCC from the vagal premigratory crest to the anterior end of the intestine. We have also investigated the function of the hand2 transcription factor that is expressed in the intestinal mesoderm-derived mesenchyme in zebrafish at early stages of intestinal development. We find that hand2 function is not required for the initial migration of vagal NNCs to the anterior end of the intestine but it is necessary for the migration/proliferation of ENS precursors along the intestine and their subsequent differentiation. We show that SHH secreted from the intestinal endoderm is necessary and sufficient to enable NCC to migrate from the premigratory vagal region to the anterior end of the intestine. We also show that SHH is subsequently required for the migration of ENS NCC along the intestine. We show that SHH acts as a mitogen and is required for the normal proliferation of vagal NCC that gives rise to the ENS precursors. Finally we present data showing that endoderm and HH signaling are required for the normal expression of gdfn in the zebrafish intestine and that the absence of gdfn expression, when HH signaling is perturbed, correlates with the lack of migration of vagal neural crest cells to and along the intestine.

Materials and methods

Zebrafish maintenance and breeding

Fish were raised and kept under standard laboratory conditions at 28.5 °C (Westferfield, 1993). Embryos were stage and fixed at specific hours or days post fertilization (hpf or dpf) as described Kimmel et al. (1995). To better visualize internal structures in some experiments embryos were incubated with 0.2 mM 1-phenoxy-2-thioura (Sigma) to inhibit pigment formation (Westferfield, 1993), handsaff (Yelon et al., 2000) fish line was obtained from Deborah Yelon and the syu (Brand et al., 1996; Schauerte et al., 1998) and smu fish lines were obtained from Philip Dilorio.

Immunohistochemistry

Embryos were processed for immunohistochemistry as previously described (Rible and Kruse, 2000). Differentiated enteric neurons were revealed with the anti-Hu mAb 16A11 (Molecular Probes) that labels differentiated neurons (Marussich et al., 1994) or by immunohistochemically staining embryos obtained from the HuC-GFP transgenic line (Park et al., 2000) that expresses GFP in differentiated neurons with a rabbit polyclonal anti-GFP antibody (Molecular Probes). Vagal neural crest cells and migrating ENS precursors were identified in FoxD3-GFP transgenic fish line (Gilmour et al., 2002; Lister et al., 2006) immunocytochemically using a mouse monoclonal anti-GFP antibody and the rabbit polyclonal anti-GFP antibody (Molecular Probes). Proliferating cells were identified using a monoclonal anti-phosphohistone H3 antibody (Upstate) (Ajiro et al., 1999). Cells undergoing apoptosis were identified using an anti-activated Caspase-3 rabbit polyclonal antibody (BD Biosciences). The anti-Hu mAb was visualized using an Alexa Fluor 568 anti-mouse IgG antibody (Molecular Probes), the rabbit polyclonal antibodies were visualized using either an Alexa Fluor 680 or an Alexa Fluor 488 anti-rabbit IgG antibody (Molecular Probes) and the mouse anti GFP antibody was visualized using an Alexa Fluor 488 anti-mouse IgG antibody (Molecular Probes).

Whole-mount in situ hybridization

Embryos were collected and processed for whole-mount in situ hybridization as previously described (Thiise et al., 1999). Dioxigenin-labeled riboprobes used in this study were synthesized from templates linearized and transcribed as follows: crestin (Rubinstein et al., 2000), sox2b (Shepherd et al., 2004), sox2b (Angelo et al., 2000) BsmHI and T7; myh11 (Wallace et al., 2005b) BsmHI T7; alpha-smooth muscle actin (α SMA) (Georgievic et al., 2007) BsmHI T7; gdfn (Shepherd et al., 2001) Not T3; ptc-1 (Concordet et al., 1996), BsmHI and T3; shh (Eiker et al., 1995). HindIII and T7; ret (Bisgrove et al., 1997), Not and T7; and fox2 (Odenthal and Nusslein-Volhard, 1998). Apol and T7. dioxigenin-labeled probes were visualized with NBT/BCIP coloration reactions. Double-label wholemount fluorescent in situ hybridization (FISH) was carried out as described (Filippi et al., 2007). Confocal z-stacks of whole-mount FISH embryos were recorded using a Zeiss LSM 510 laser scanning confocal microscope. Cross-sections of in situ hybridized embryos were made by embedding embryos in 1 ml gelatin-albumin (25 mg gelatin, 1.3 g BSA, 0.9 g saccharose, in 4.5 ml PBS) for 1 h at room temperature. The gelatin-albumin was then replaced with 1 ml of gelatin-albumin + 35 μl of 50% glutaraldehyde. To harden the embedding mixture embryos were incubated at 4 °C overnight. Sections were cut with a Leica vibrotome at 10–20 μm.

Embryonic microinjections

shh mRNA was synthesized using the mMessage mMachine kit (Ambion) and injected at a concentration of 150 ng/μl. Approximately 4 nl of diluted mRNA was injected into 1- to 2-cell embryos using a gas-driven microinjection apparatus (PV820 WPI) through a micropipette.

sox32 (cazonova) morpholino antisense oligonucleotide (Gene Tools) was designed to the previously described morpholino translation blocking sequence (Dimitrievs et al., 2001). hand2 morpholino was designed to target the translation start site of the gene to the following sequence: 5′ CCTCCACTAAACTCATGCGACACG 3′.

The morpholino phenocopied the hand2 zebrafish deletion mutant (Yelon et al., 2000). A 5 A-base pair mismatch hand2 control morpholino failed to illicit any phenotype. The oligos were resuspended in sterile filtered water and diluted to working concentrations in a range between 1 and 5 μg/ml. Approximately 1 nl of diluted morpholino was injected into 1- to 2-cell embryos using a gas-driven microinjection apparatus.

Cyclopamine treatment

Cyclopamine (Toronto Research Company) was dissolved in 100% ethanol to generate a 10 mM stock solution. Subsequently this stock solution was further diluted in embryo media to give a working concentration of 10 μM. Wild type embryos were soaked in the cyclopamine containing embryo medium at 28 °C for 24–36 hpf or 36–60 hpf after which the embryo media was replaced with fresh embryo media that did not contain cyclopamine. Embryos were then incubated until they were fixed and processed for immunohistochemistry or in situ hybridization.

Transplantation experiments

Wild-type donor embryos used in transplant experiments were from an AB/TL strain. Host embryos were generated from an in cross of syu or smu heterozygotes. Donor embryos at the 1- to 2-cell stage were injected with 1.2 ng of sox32 mRNA combined with 25 μM tetramethylrhodamine lyseine f hopeful biotin dextran (10 mM, Molecular Probes) to convert most of the donor cells to an endodermal cell fate (Holzschuh et al., 2005; Stafford et al., 2006); sox32 mRNA was synthesized using the mMessage mMachine kit (Ambion) as described above. At the stage level, 20-40 donor cells were transplanted into unlabelled sibling host embryos. Embryos were then cultured in embryo medium with 10 U/ml penicillin and 10 U/ml streptomycin. Host embryo ENS development was determined in 60 hpf embryos by phox2b in situ hybridization. Donor cells from the transplant were detected in hosts using an avidin-biotinylated complex (ABC kit, Vectastain) and a DAB substrate. Embryos were embedded and sectioned as described in the wholemount in situ hybridization section. Genotypes of the host embryos were determined by phenotypic appearance typical for shh−/−, smu−/− or wild type embryos.
Results

Requirements for endoderm and mesodermal hand2 expression in zebrafish ENS development

In zebrafish ENS precursors first migrate from the post-otic (vagal) region to the anterior end of the intestine beginning around 30 hpf and continue to do so until 40 hpf. At this stage the intestine is little more than a rod of endoderm that has not yet formed a lumen (Ng et al., 2005; Wallace et al., 2005b; Wallace and Pack, 2003). A thin layer of loosely associated mesodermal-derived mesenchyme surrounds the endoderm that will form the smooth muscle of the intestine beginning around 60 hpf (Georgijevic et al., 2007; Wallace et al., 2005a). Between 34 and 36 hpf phox2b expressing ENS precursors can be first observed closely associated with the anterior end of the intestinal endoderm (Elworthy et al., 2005; Shepherd et al., 2004). Subsequently ENS precursors migrate as two symmetric streams either side of the rod of endoderm reaching the distal end of the intestine.

**Fig. 1.** Endoderm and mesodermal hand2 function are required for normal ENS and intestinal smooth muscle development but hand2 is not required for the initial migration of vagal neural crest to the anterior end of the intestine and endoderm is not required for mesodermal hand2 expression. (A, D, G, M, O) wild-type embryos; (B, E, H, K, N, P) cas/sox32 morphant embryos and (C, F, I, L) hand2 morphants. (A–C) Ventral view of the vagal region of 36 hpf embryos that have been hybridized with riboprobes for crestin showing a failure of vagal NCC migration to the anterior end of the intestine in sox32 morphants but with no effect on these NCC in hand2 morphants. (D–F) Lateral view of 60 hpf embryos that have been hybridized with riboprobes for phox2b showing a failure of phox2b expressing cells to populate the entire length of the intestine in cas/sox32 and hand2 morphants. (G–I) Closeup view of the intestine of 60 hpf embryos that have been hybridized with riboprobes for phox2b and phox2b expressing cells to populate the entire length of the intestine in cas/sox32 and hand2 morphants. (J–L) Closeup view of the intestine of 72 hpf embryos whole-mount in situ hybridized embryos that have been hybridized with riboprobes for αSMA. Black boxes in panels D–F are the regions that are shown in close up in panels G–I. Arrowheads (M) indicate the αSMA expressing cells in the intestine. (O, P) Ventral view of 30 hpf whole-mount in situ hybridized embryos that have been hybridized with an antisense probe for hand2. Arrows (A, C) indicate the migrating enteric precursors. White arrowheads (G, I) indicate phox2b expressing cells in the intestine. Black arrowheads (J, L) indicate myh11 expressing cells in the intestine. Black arrowheads (M) indicate the αSMA expressing cells in the intestine. Anterior is to the left.
sox32 morphants exhibit cardiabi morphants at 36 hpf with probes for sox32 (casanova) premigratory vagal crest region to the ventral midline, we hybridized ENS development for the migration of ENS precursors from the premigratory vagal neural crest to the anterior end of the intestine just prior to the stage when ENS NCCs are migrating along the intestine (Pietsch et al., 2006) (Fig. 1). To determine whether endoderm is required earlier in migration of the ENS precursors from the premigratory vagal neural crest to the anterior end of the intestine, we examined the expression of the smooth muscle markers myh11 and alpha smooth muscle actin (αSMA) are severely perturbed or absent in sox32 morphant embryos (Figs. 1, K, M, N). By contrast expression of the lateral plate mesoderm marker hand2 is not perturbed in sox32 morphants (Figs. 10, P).

We next investigated the function of hand2 in the development of zebrafish ENS. In the zebrafish intestine hand2 is expressed in the lateral plate mesoderm-derived mesenchyme at stages prior to and during the period when ENS NCCs are migrating along the intestine (Figs. 10 and 2B). We hypothesized that zebrafish hand2 has functions in intestinal development that include those that are normally carried out by hand1 in mouse. Mouse hand1 is expressed in intestinal mesenchyme whereas mouse hand2 is not (D’Autreaux et al., 2007). We therefore determined the effect of morpholino knockdown of hand2 on intestinal smooth muscle development and ENS NCC migration. We also determined whether the hand2 morphant intestinal smooth muscle and ENS phenotypes were the same as that in handsoff mutant fish that have a deletion of the hand2 gene (Yelon et al., 2000). Consistent with our hypothesis, handsoff mutant embryos and hand2 morphant embryos fail to express the intestinal smooth muscle markers myh11 at 60 hpf (Fig. 1L). In addition ENS precursors fail to migrate along the intestine at 60 hpf in hand2 morphants as determined by phox2b in situ (Figs. 1F, I). Subsequently there is a significant decrease in the number of differentiated ENS neurons at 96 hpf in morphants as determined by Hu immunoreactivity (Fig. 2E). To determine if the initial migration of ENS precursors from the premigratory vagal neural crest to the anterior end of the intestine is also perturbed in hand2 morphants we fixed embryos at 36 hpf and examined crestin expression. In contrast to the sox32 morphants, migration of the ENS precursors to the anterior end of the intestine is not perturbed in the hand2 morphants (Figs. 1B, C). Taken together these experiments suggest that both endoderm and hand2 are required for normal ENS and intestinal smooth muscle development in zebrafish. However only endoderm is required for the migration of ENS precursors from the premigratory vagal neural crest region to the anterior end of the intestine.

Expression of Sonic hedgehog and hedgehog signaling pathway components during ENS development

Previous studies have shown that SHH acts as a mitogen in vitro for purified enteric neural crest cells and in turn modulates the ENS NCC migratory stimulating activity of GDNF (Fu et al., 2004). Furthermore, previous studies in mouse and avian have shown expression of shh correlates with ENS development and genetic or pharmacological perturbation of HH activity in the intestine causes ENS patterning defects (Ramalho-Santos et al., 2000; Sukegawa et al., 2000).

To determine if shh and a component of its receptor, patched-1 (ptc1), have expression patterns that correlate with the migration of ENS NCC precursors from the premigratory vagal crest region to the anterior end of the intestine and along it, we examined the expression patterns of these genes. The location and distribution of ENS NCC was determined in age-matched embryos/sections using crestin or phox2b. shh expression is first detected at the anterior end of the intestine around 30 hpf (Figs. 3A, B). Expression is restricted to this anterior region of the intestine just prior to the stage when ENS NCC first migrate from the vagal premigratory neural crest to this part of the gut. This pattern of expression continues through 36 hpf, the stage at which the chain of ENS precursors migrating to the anterior end of the intestine can be clearly seen (Fig. 1A). ptc1 is widely expressed throughout the mesenchyme adjacent to the endoderm and is expressed by ENS NCC (Figs. 3G–L). This pattern of shh and ptc1 expression is consistent with SHH having a direct role in zebrafish ENS development.

ENS development is perturbed in hedgehog signaling pathway mutants and in cyclopamine treated embryos

To determine the functional role of SHH in zebrafish ENS development, we examined the expression of ENS NCC markers phox2b, ret and crestin in the hedgehog pathway homozygous mutants sonic you (syu), that has a deletion in the shh gene (Brand et al., 1996; Schauerte et al., 1998), and smooth muscle-omitted (smu), that has a
Fig. 3. The expression pattern of shh and ptc-1 correlate with the development of ENS. (A, B, E, F) Wholemount embryos hybridized in situ with a shh antisense probe at the indicated developmental stages. (C, D, H) Wholemount embryos hybridized in situ with a ptc-1 antisense probe at the indicated developmental stages. (G) 60 hpf wholemount in situ hybridized embryos hybridized with a phox2b antisense probe to reveal the distribution of the ENS NCC in the intestine. (I) Cross-section taken through a 60 hpf embryo hybridized with a phox2b antisense probe to reveal the distribution of ENS NCC in the intestine. (J) Cross-section taken through a 60 hpf embryo hybridized with a ptc-1 antisense probe. At all stages examined, shh and ptc-1 are expressed in regions that correlate with the development of the ENS. Furthermore, comparison of the pattern of ptc-1 expression in the intestine at 60 hpf shows that phox2b expressing ENS NCC are located in the ptc-1 expression domain. (K–K″) Comparison of the pattern of ptc-1 expression (K′, green channel) to that of phox2b (K, red channel) in the intestine at 42 hpf by double fluorescent in situ hybridization shows that phox2b expressing ENS NCC co-express ptc-1 (K″, merge channels). Expression was documented by confocal stacks of images (L–L″). Ventral view of the vagal region of a 36 hpf wildtype embryo showing a comparison of the pattern of ptc-1 expression (L′, green channel) to that of phox2b (K, red channel) by double fluorescent in situ hybridization showing pre-enteric ENS NCC’s express ptc-1 (K″, merge channels). (A) Lateral view of embryo. (B, D, F, G, H, K–K″, L–L″) Ventral views of embryos with the yolk removed. (C, E) Dorsal views of embryo. not (A, B, C, E, F) indicates notochord. end (A–C) indicate endoderm. * (D, G, H, I, J) indicates the gut lumen. Arrows (K–K″) indicate ptc-1 phox2b coexpressing cells. The cells delineated by the white line in (L, L″) are the migrating pre-enteric ENS NCC. Scale bar (K″, L″) is 20 μm. In all wholemounts (A–H) anterior is to the left.
mutation in zebrafish *smoothed (smo)* which completely abolishes all HH signaling (Barresi et al., 2000; Chen et al., 2001; Varga et al., 2001). At 60 hpf both *syu* and *smu* mutants completely lack any ENS precursors along the length of the intestine as compared to wild type controls (Figs. 4A–C). To determine if the initial migration of ENS precursors to the anterior end of the intestine is similarly perturbed in these mutants, the pattern of *ret* and *crestin* expression was examined in 36 hpf *syu* and *smu* mutants as compared to wildtype embryos. No migrating ENS NCC’s were observed in either mutant using either marker (Figs. 4D–I).

To further define the precise temporal requirements for HH signaling in normal ENS development we treated wildtype zebrafish embryos with cyclopamine at specific time points during embryogenesis. Cyclopamine is a steroidal alkaloid that inhibits activation of the HH signaling pathway by directly binding to Smo (Chen et al., 2002; Cooper et al., 1998; Incardona et al., 1998). In addition, this experiment also determined if the absence of ENS observed in the *syu* and *smu* mutants is a secondary phenotype, due to the loss of HH signaling at earlier stages of embryogenesis that perturbed vagal crest specification and patterning. Previously it has been shown that HH signaling is required for survival, migration and patterning of cranial neural crest populations in xenopus, avian and zebra fish (Ahlgren and Bronner-Fraser, 1999; Barresi et al., 2000; Brand et al., 1996; Brito et al., 2006; Charrier et al., 2001; Dunn et al., 1995; Kimmel et al., 2001; Varga et al., 2001; Wada et al., 2005). However HH signaling is not directly required for neural crest formation in zebrafish (Ungos et al., 2003). To determine the precise temporal requirement for HH signaling in ENS development, embryos were treated with cyclopamine either from 24–36 hpf, during the pre-enteric phase of ENS development, or from 36–60 hpf, referred to as the enteric phase of ENS development in this paper. The embryos were then fixed at 60 hpf and process for in situ hybridization with a *phox2b* probe to determine if the pattern of ENS precursor migration along the intestine was perturbed. The pattern of intestinal smooth muscle development was also assessed at 60 hpf, based on *myh11* expression and at 72 hpf based on α SMA expression. Other cyclopamine treated embryos were fixed at 96 hpf to determine the state of differentiation of ENS neurons by anti Hu immunohistochemistry.

Cyclopamine treatment caused an almost complete loss of ENS precursors along the intestine in 24–36 hpf treated embryos as compared to controls (Figs. 5A, B). A similar though slightly less severe reduction in the number of ENS precursors was seen in 36–60 hpf treated embryos (Fig. 5C). Under both experimental conditions the small number of *phox2b* expressing precursors remaining were restricted to the anterior end of the intestine up to somite 7. Similarly at 96 hpf no differentiated enteric neurons could be detected along the length of the gut in 24–36 hpf cyclopamine treated embryos, while only a very few neurons could be seen in 36–60 hpf cyclopamine treated embryos as compared to control embryos (Figs. 5G–I).

Consistent with previous experiments examining the affect of temporal treatment of cyclopamine on DRG development we observed varying affects on DRG number and size depending on the period of cyclopamine treatment (Ungos et al., 2003). Surprisingly expression of the intestinal smooth muscle markers *myh11* at 60 hpf and α SMA at 72 hpf were equally perturbed in both 24–36 hpf and 36–60 hpf treated embryos (Figs. 5D–I). However expression of *myh11* was less affected in the pharyngeal arches in 24–36 hpf embryos than the 36–60 hpf treated embryos.

To determine if the initial migration of ENS precursors from the vagal premigratory crest to the anterior end of the intestine is perturbed in the 24–36 hpf treated embryos we fixed treated embryos at 36 hpf and stained with *crestin*. At 36 hpf no chevron of ventromedial migrating vagal ENS NCC could be detected in the 24–36 hpf cyclopamine treated embryos (Fig. 6). Taken together these data suggest that there are two phases of HH signaling required for normal ENS development in zebrafish. HH signaling is required in a pre-enteric phase when the ENS NCC precursors migrate to the anterior end of the intestine between 24 and 36 hpf. Subsequently HH signaling is required from 36–60 hpf when the ENS NCC precursors migrate along the intestine.

Proliferation of vagal NCC and ENS precursors is decreased in cyclopamine treated embryos

To determine the mechanism that causes the failure of the ENS NCC to populate the intestine in cyclopamine treated embryos we examined whether there is an increase in apoptosis or a decrease in proliferation in treated embryos.

To assess if there is an increase in apoptosis in cyclopamine treated embryos we determined the pattern of activated Caspase 3 immunoreactivity in vagal NCC and ENS precursors at 30 hpf and 48 hpf in control and experimental embryos. We used *foxD3::gfp* embryos for these studies as they express green fluorescent protein (GFP) in vagal NCC and ENS precursors (Gilmour et al., 2002; Lister et al., 2006). No increase in apoptosis was observed in these cells in cyclopamine treated embryos.
treated embryos (Figs. 7A, C data not shown). This suggests that apoptosis is not the mechanism that causes the loss of ENS precursors in cyclopamine treated embryos.

We next investigated whether there is a decrease in proliferation in the vagal NCC and ENS precursors due to the cyclopamine treatment. 30 hpf cyclopamine treated and control foxD3∷gfp transgenic embryos were immunocytochemically stained with an anti-phosphohistone H3 antibody to identify proliferating cells (Ajiro et al., 1996; Pietsch et al., 2006). A statistically significant decrease was observed in the number of proliferating vagal NCC's and ENS precursors in cyclopamine treated embryos as compared to controls (Figs. 7B, D). Only 6.8±4.3% of vagal NCC and ENS precursors were seen to be proliferating in cyclopamine treated embryos vs. 21.2±5.1% in control embryos (n=5). This statistically significant result (P=0.002 using Student’s t-test) suggests that reduced proliferation of the vagal NCC causes the loss of ENS precursors in the intestine and is consistent with recent data from chick that suggests cell proliferation drives neural crest cell invasion of the intestine (Simpson et al., 2007).

The number of ENS precursors in the intestine is increased by over expression of Sonic hedgehog

Given that a loss of HH signaling caused a reduction in ENS precursors, we wanted to determine if overexpression of SHH early in
embryogenesis would cause an increase in the number of ENS precursors. Previous in vitro studies have shown that SHH acts as a mitogen for purified ENS NNCs (Fu et al., 2004). To determine if SHH has a mitogenic affect in vivo on the ENS precursors we injected embryos with shh mRNA at the one cell stage. The number of ENS precursors was then determined in these injected embryos by phox2b expression at 72 hpf. Overexpression of SHH consistently led to an increase in the number of phox2b expressing precursors in the intestine, with 33±2.5 phox2b expressing precursors being present in the intestine of shh injected embryos vs. 24±2 in controls (Fig. 8).

Transplantation of wild type endoderm into sonic you mutants rescues ENS development

To further demonstrate that endoderm-derived SHH is necessary and sufficient for normal ENS development, we took a genetic chimeric approach to introduce wildtype cells that express SHH into the endoderm of syu and smu mutants to determine if these wildtype cells could rescue ENS development. Wildtype cells were transplanted into the syu and smu mutants at the early blastula stage. The wild type cells were targeted to the endoderm as the donor embryos were injected at the one cell stage with sox32 mRNA along with a rhodamine biotinylated dextran lineage tracer (Holzschuh et al., 2005; Stafford et al., 2006). Experimental embryos were allowed to develop to 60 hpf at which time the development of the ENS was determined by phox2b expression. In contrast to syu−/− embryos that have no phox2b precursors in the intestine at 60 hpf (Fig. 4B) in 100% of the experimental embryos where wild type cells were transplanted into syu hosts, phox2b expressing ENS could be detected adjacent to the intestine (Figs. 9A–E) (2 independent experiments with 7/7 and 22/22 syu−/− embryos with transplanted wt endoderm). By contrast, no phox2b precursors could be detected in the intestines of smu mutants that received wildtype endodermal cell transplants (Fig. 9F) (8/8 smu−/− embryos with wildtype endoderm no phox2b expression could be detected in the intestine.

Recent in vitro studies have shown that SHH acts to promote proliferation of ENS precursors while modulating responsiveness of ENS NCC’s to the migratory stimulating activity of GDNF (Fu et al., 2004). Previously, GDNF has been shown to be a chemoattractant for ENS NCC’s in vitro and regulates ENS proliferation (Gianino et al., 2003; Natarajan et al., 2002; Young et al., 2001). Furthermore, knockdown of gdnf and its receptor complex causes an ENS NCC migration defect in zebrafish (Shepherd et al., 2001, 2004). To determine if the defects in ENS precursor migration observed in sox32 morphants, hand2 morphants and cyclopamine treated embryos were associated with changes in gdnf expression, we assessed gdnf’s expression at 36 and 60 hpf in these experimental embryos.

In sox32 morphant embryos and 24–36 hpf cyclopamine treated embryos there is no expression of gdnf at the anterior end of the intestine at 36 hpf (Figs. 10C, D). By contrast hand2 morphant embryos have a comparatively normal expression of gdnf at the anterior end of the intestine at this age (Fig. 10B). This pattern of gdnf expression in the hand2 morphants is consistent with the normal pattern of ENS NCC migration from the vagal region to the anterior end of the intestine that we see in these morphants (Fig. 1C). At 60 hpf, when
Enteric neurons (A, C, D, E); white arrows point to transplanted endoderm (C).

Expression of gdnf suggests that endoderm and HH signaling are required for enteric ganglia formation in syu mutants (Fig. 10). Cross-sections taken through 72 hpf embryos. (A-D) Ventral views with anterior to the left of 72 hpf embryos. In wild type embryos phox2b expressing enteric neurons are located on the lateral edge of the gut tube (A). No phox2b expressing cells are found around the gut of syu mutant embryos (B). Transplanted sox32 injected cells are recruited to the endoderm of syu mutants and rescue the enteric ganglia formation (C–E). Endodermal transplants from sox32 injected wild type embryos are not able to rescue enteric ganglia formation in smu mutants (F). Black arrows indicate to phox2b expressing enteric neurons (A, C, D, E), white arrows point to transplanted endoderm (C–F).

Hand2 is normally expressed along the complete length of the intestine, sox32 morphant embryos and cyclopamine treated embryos have no gdnf expression along the length of the gut (Figs. 10K, M, N, O). As at 36 hpf, the pattern of gdnf expression at 60 hpf is comparatively normal in hand2 morphants (Fig. 10L). Taken together these data suggest that endoderm and HH signaling are required for gdnf expression in the zebrafish intestine while hand2 is not. Moreover the ENS defect observed in hand2 morphants at 60 hpf and 96 hpf is not due to defects in gdnf expression.

Discussion

Our results provide evidence that endoderm-derived SHH/hedgehog signaling is specifically required in two distinct phases of ENS development: i) a pre-enteric phase that is necessary for the normal migration of zebrafish ENS NCC precursors from the vagal crest to the anterior end of the intestine ii) an enteric phase that is required for the ENS NCC migration along the intestine. We show that proliferation of vagal NCC and ENS NCC is reduced when HH signaling is perturbed and that SHH acts as a potent mitogen for ENS NCC in vivo. We show that zebrafish hand2 function is not required in the pre-enteric phase of ENS development but is required for enteric stages of ENS development. Furthermore we show that hand2 function is required for the initial stages of development for intestinal muscle suggesting that zebrafish hand2 has functions in ENS/intestinal development that normally require hand1 in other species. Finally, we show that in vivo perturbation of either endodermal development or HH signaling results in loss of intestinal gdnf expression. These studies add to the previously described roles of HH signaling in neural crest and ENS development and provide evidence for an early requirement for HH signaling in ENS development in a pre-enteric phase. We also provide in vivo evidence that endoderm-derived HH signaling modulates gdnf expression in the intestine while hand2 does not.

Role of endoderm in ENS development

In zebrafish, vagal neural crest-derived ENS precursors first migrate towards the anterior end of the intestine between 30 and 36 hpf forming a distinct chain of migrating precursors. Previously we have demonstrated a requirement for endoderm for the anterior–posterior migration of the ENS precursors along the length of the intestine (Pietsch et al., 2006). While the central role of endoderm in ENS development is not surprising, the requirement for endoderm in the ventromedial migration of the vagal crest-derived ENS NCC, as

Fig. 9. Transplanted wild type endoderm rescues the enteric ganglia in syu mutants. (A–D) Ventral views with anterior to the left of 72 hpf embryos. In wild type embryos phox2b expressing enteric neurons are located on the lateral edge of the gut tube (A). No phox2b expressing cells are found around the gut of syu mutant embryos (B). Transplanted sox32 injected cells are recruited to the endoderm of syu mutants and rescue the enteric ganglia formation (C–E). Endodermal transplants from sox32 injected wild type embryos are not able to rescue enteric ganglia formation in smu mutants (F). Black arrows indicate to phox2b expressing enteric neurons (A, C, D, E), white arrows point to transplanted endoderm (C–F).

Fig. 10. Expression of gdnf in the intestinal mesenchyme requires endoderm and HH signaling but not hand2 function. (A, F, K) Wildtype control embryos. (B, G, L) Hand2 morphants (C, H, M) mit/sox32 morphant embryos, (D, I, N) 24–26 hpf cyclopamine treated embryos and (E, J, O) 36–60 hpf cyclopamine treated embryos. (A–E) Ventral views of 36 hpf wholemount embryos that have been hybridized with a gdnf antisense probe and the yolk removed. In sox32 morphant (C) and 24–36 hpf cyclopamine treated embryos (D) there is a loss of gdnf expression at the anterior end of the intestine. (F–J) lateral view of 60 hpf wholemount embryos, that have been hybridized with a gdnf antisense probe, showing the loss of intestinal gdnf expression in sox32 morphants and cyclopamine treated embryos. (K–O) lateral view of the intestine of 60 hpf wholemount embryos that have been hybridized with a gdnf antisense probe. Arrows (A, B, E) indicate gdnf expression at the anterior end of the intestine. Arrow heads (K, L) indicate the gdnf expression along the length of the intestine. Anterior is to the left.
revealed by the loss of the chain of these cells in sox32 morphants, is novel. To date the tissues, mechanisms and molecules involved in patterning the migration of the vagal ENS NCCs to the anterior end of the intestine have not been directly addressed. By determining that the endoderm is the key signaling center for this initial migration to occur, we have been able to focus on secreted molecules produced by this tissue that are responsible for this activity. This has led us to determine that SHH is an essential molecule for this migration to occur.

**Evolutionary differences in hand2 function in zebrafish as compared to mouse**

In most vertebrate species there are two hand genes. In mice and avian hand2 is expressed in the developing ENS while hand1 is expressed in the mouse intestinal mesenchyme (Cserjesi et al., 1995; D’Autreaux et al., 2007; Hendershot et al., 2007; Wu and Howard, 2002). By contrast in zebrafish there is only a single hand gene. At the sequence level the zebrafish hand gene is more orthologous to hand2 than hand1 (Angelo et al., 2000). However the expression of zebrafish hand2 in the intestine is more like that of hand1 in mouse at early stages of intestinal development as it is expressed in the intestinal mesenchyme (Angelo et al., 2000; D’Autreaux et al., 2007) (Fig. 1). At later stages of intestinal development zebrafish hand2 appears to be expressed in both the intestinal mesenchyme and ENS precursors (Fig. 2). In mouse, hand1 is expressed in the intestinal muscle cells and is required for vascular smooth muscle development while hand2 is exclusively expressed in enteric neurons and ENS NCC (D’Autreaux et al., 2007; Morikawa and Cserjesi, 2004). The function of Hand1 has not been addressed in mouse intestinal muscle development due to the early embryonic lethality of the Hand1 null mouse at E9–9.5 (Morikawa and Cserjesi, 2004). However, murine Hand2 has been shown to be required for normal differentiation of enteric neurons but not for the migration of ENS precursors along the intestine (D’Autreaux et al., 2007; Hendershot et al., 2007). Our data raises the possibility that in zebrafish hand2 is responsible for the biological activities of both hand1 and hand2 found in other species. Future studies will determine whether this is the case.

**Function of Sonic hedgehog signaling on ENS development**

Our results demonstrating the loss of ENS precursors in HH pathway mutants and cyclopamine treated embryos, as well as the increase in number of ENS NCC in SHH overexpressing embryos, is consistent with previous studies that have examined the effect of SHH on ENS NCC (Fu et al., 2004). Our results also support the findings from other studies that have demonstrated that different axial populations of NCC respond differently to SHH.

SHH has been shown to play an important role in survival of both neural tube and cranial neural crest. Injections of function blocking anti-SHH antibody into chick cranial mesenchyme results in a loss of branchial arch structures and is associated with significant cell death in both the neural tube and the neural crest (Ahlgren and Bronner-Fraser, 1999). More recent chick studies have demonstrated that ventral foregut endoderm-derived SHH is specifically required at early stages of jaw development, between somite stages 5 and 7, for the survival of branchial arch 1 NCC (Brito et al., 2006). Similarly, cyclopamine treatment of Xenopus embryos results in a reduction of craniofacial cartilages and promotes cell death in explants of cranial neural crest (Dunn et al., 1995). More recent mouse studies, in which smo was genetically removed from migratory neural crest, have also demonstrated a requirement for hedgehog signaling in craniofacial and cardiac NCC survival (Goddeiris et al., 2007; Jeong et al., 2004). In contrast to cranial NCC, when HH signaling is perturbed during dorsal root ganglion (DRG) development in avian, Xenopus or zebrafish, there is no obvious increase in cell death within trunk crest (Ahlgren and Bronner-Fraser, 1999; Dunn et al., 1995; Ungos et al., 2003). This has led to the suggestion that the anti-apoptotic effect of SHH is restricted to cranial neural crest populations. However we do not see any obvious increase in cell death in the premigratory vagal neural crest that gives rise to the ENS NCC when HH signaling is perturbed (Figs. 7A, C), though we cannot definitively rule out that there is no cell death in these cells as we have no specific markers for the ENS precursors in the premigratory NCC that could reveal this. The apparent lack of cell death in the ENS NCC precursors though is consistent with data from mouse In vitro studies (Fu et al., 2004). In these studies no difference was observed in the number of apoptotic cells in ENS NCC-derived neurospheres cultured in the presence or in the absence of SHH (Fu et al., 2004).

Our finding that SHH is necessary for ENS NCC migration to the anterior end of the intestine from the vagal region in the pre- enteric phase of ENS development is novel and significant. This result is somewhat consistent with other studies that have shown SHH has a role in determining the pattern of migration of a number of other neural crest populations. In mice SHH signaling is required for the normal pattern of migration of cardiac and trunk neural crest (Washington Smoak et al., 2005). Similarly HH signaling is required for the normal pattern of migration of trunk NCC DRG precursors in zebrafish, as revealed by HH pathway mutants (Ungos et al., 2003). In addition SHH determines the pattern of migration of cranial NCC that form the anterior neurocranium in zebrafish (Wada et al., 2005). While it is possible that SHH could act as a chemoattractive cue for ENS precursors from the vagal premigratory NCC to the anterior end of the intestine our results instead show that HH regulates the proliferation of the vagal NCC. As a result the ENS defect in cyclopamine treated embryos appears to arise due to there being insufficient ENS precursors generated within the premigratory vagal NCC when HH signaling is perturbed. This finding is consistent with recent studies that show NCC proliferation drives the invasion of ENS precursors along the length of the chick intestine (Simpson et al., 2007). We now extended this model and suggest that proliferation drives the pre-enteric migration of ENS NCC precursors form the vagal premigratory NCC region to the anterior end of the intestine.

During the enteric phase we propose that SHH continues to act as a mitogen for ENS precursors and drive their migration along the intestine, as well as acting as a morphogen patterning the intestinal mesenchyme. This in vivo mitogenic activity of SHH for ENS NCC is a comparatively novel finding. Previously SHH has been shown to be a mitogen for ENS neurospheres in vitro (Fu et al., 2004). We now show by shh RNA over-expression a similar mitogenic activity in vivo, however we cannot rule out the possibility that this activity is an indirect effect and is due to the increased secretion of other mitogens from the intestinal mesenchyme rather than SHH acting directly as an ENS NCC mitogen. The putative increased secretion of other mitogens could result from the morphogenetic actions of SHH on the intestinal mesenchyme. Previous studies in mouse and chick have demonstrated that perturbation of HH signaling results in intestinal and ENS patterning defects (Ramalho-Santos et al., 2000; Sukegawa et al., 2000). These studies demonstrated that SHH is the key factor that patterns the radial axis of the intestine and led to the proposal that SHH inhibits smooth muscle differentiation in the intestine (Ramalho-Santos et al., 2000; Sukegawa et al., 2000). A number of previous studies have shown that SHH secreted from the endodermal epithelium induces expression of BMP4 in the adjacent non-smooth muscle mesenchyme (Narita et al., 1998; Roberts et al., 1995, 1998; Sukegawa et al., 2000). More recent studies have suggested that down regulation of BMP signaling in the intestinal mesenchyme is also required for intestinal smooth muscle differentiation (De Santa Barbara et al., 2005). Our data shows that blocking SHH signaling with cyclopamine results in a failure of intestinal smooth muscle differentiation in the intestinal mesenchyme of zeb-
rafish, counter to what would have been predicted from the previous models. This suggests that other factors are involved in the intestinal smooth muscle differentiation, rather than just a simple down regulation/inhibition/lack of BMP signaling, and a SHH dependent myogenic factor is required to induce *myh11* and *α SMA* expression in zebrafish.

As SHH patterns the radial axis of the intestine it will also indirectly act as an ENS NCC guidance cue for the migrating precursors within the intestine. As previously mentioned, SHH induces BMP expression in the intestinal mesenchyme. Recently BMP signaling has been shown to regulate PSA-NCAM levels on NCC (Fu et al., 2006). Increased BMP signaling leads to the increased addition of PSA to NCAM. The increased amount of PSA-NCAM on the cell-surface of the ENS NCC in turn inhibits their migratory ability. As cyclopamine treatment should cause a decrease in BMP expression in the intestinal mesenchyme, we would predict that if this model also applies to zebrafish, there would be an increase in the migratory behavior of the ENS precursors due to reduced PSA-NCAM. However this does not appear to be the case based on our in situ analysis. We propose that the key activity of SHH on ENS precursors, during the enteric phase of ENS development, is as a mitogen rather than as an indirect regulator of ENS precursors migratory ability. We believe that when HH signaling is perturbed during this enteric phase the reduced/lack of proliferation of the ENS NCC precursors in the intestine will mask any changes in their migratory behavior.

In addition to BMP’s role in regulating the migratory behavior of ENS NCC along the intestine, GDNF has been shown in avian, mouse and zebrafish to be critical for stimulating the migratory behavior of these precursors along the intestine (Natarajan et al., 2002; Shepherd et al., 2001, 2004; Young et al., 2001). Furthermore, SHH has been shown to regulate GDNF’s migratory stimulatory behavior for ENS NCC’s in vitro while GDNF acts to inhibit SHH’s mitogenic activity (Fu et al., 2004). Our data is consistent with SHH regulating GDNF’s activities in ENS development, as perturbation of endoderm development or perturbation of SHH signaling results in a loss of *gdnf* expression in the intestinal mesenchyme. Our results are also consistent with our previous studies that show knockdown of *gdnf* or its receptor complex perturbs the migration of ENS precursors along the intestine (Shepherd et al., 2001, 2004). Strikingly though, when we knockdown expression of *gdnf* or its receptor, we do not see any perturbation in the pre-enteric migration of ENS NCC from the vagal crest to the intestine (Shepherd et al., 2001, 2004). As a result SHH must be acting either directly or indirectly, via the production of some unknown chemoattractant, to direct the vagal crest-derived ENS NCC to the anterior end of the intestine. Future studies will attempt to determine what the molecular basis of this activity is. These studies will also address whether SHH acts directly or indirectly on the ENS precursors in the enteric phase of ENS development. Current genetic chimera techniques do not effectively target ENS precursors due to the small number of ENS NCC in the premigratory vagal neural crest. We are currently developing techniques that will more effectively target these cells. We will also determine if SHH directly regulates GDNF expression. What is clear is that there is a careful balance between the GDNF, HH and BMP signaling pathways in the development of the intestine and the ENS and if this balance is perturbed it results in intestinal patterning defects as well as intestinal aganglonosis.

In summary, our analysis of the role of endoderm-derived SHH in the development of the ENS has demonstrated that HH signaling is required in two phases, a pre enteric and an enteric phase for normal ENS development (Fig. 11). We show that HH signaling regulates migratory behavior and proliferation of the ENS precursors in vivo. We have also clarified the role of *hand2* in zebrafish intestinal development by demonstrating that the single zebrafish *hand* gene has functions correlated with both *hand* genes found in other vertebrates. Finally, we show that endoderm and HH signaling regulates *gdnf* expression in the intestine, highlighting a central role of endoderm and SHH in patterning the intestine and the ENS.

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