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Review article

Enteric nervous system development in avian and zebrafish models

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ABSTRACT

Our current understanding of the developmental biology of the enteric nervous system (ENS) and the genesis of ENS diseases is founded almost entirely on studies using model systems. Although genetic studies in the mouse have been at the forefront of this field over the last 20 years or so, historically it was the easy accessibility of the chick embryo for experimental manipulations that allowed the first descriptions of the neural crest origins of the ENS in the 1950s. More recently, studies in the chick and other non-mammalian model systems, notably zebrafish, have continued to advance our understanding of the basic biology of ENS development, with each animal model providing unique experimental advantages. Here we review the basic biology of ENS development in chick and zebrafish, highlighting conserved and unique features, and emphasising novel contributions to our general understanding of ENS development due to technical or biological features.

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1. Introduction

It is clear from the scope of presentations at the 4th International Symposium on “Development of the Enteric Nervous System: Cells, Signal, Genes and Therapy”, held in Rotterdam, The Netherlands (19–22 April, 2015) that active research using model systems is fuelled in equal measure by a fascination with the basic biology of the enteric nervous system (ENS) and the drive to model and unravel the genetic basis of ENS disease states.

The use of model systems to study ENS development is longstanding. For more than 20 years, studies in mouse have been critical to understand genetic control of ENS development and to model ENS diseases. However, these studies were preceded by work using other model systems, notably the chick. For example, study of avian systems initially described the neural crest origins of the ENS, and provided a framework for understanding phenotypes arising in newly generated mouse mutants. More recently, studies in these and other non-mammalian model systems, such as zebrafish, are being used to model ENS development and ENS diseases (Fig. 1). Technical innovations have meant that there has been an ever-increasing capacity to perform genetic analysis with these alternative systems, making them increasingly used and

increasingly important.

In this review we will describe key features of ENS development in chick and zebrafish, and will highlight important similarities and differences between these systems and compare to mammalian systems. Novel contributions to our general understanding of ENS development made by studies in these model systems, especially when due to unique biological traits or technical capacities, will be emphasized. The unique experimental tools available in these different model organisms will be highlighted. Finally, we will consider the future scope for use of model systems, to more fully understand ENS biology and ENS disease states.

2. The chicken embryo as a model for ENS development

The avian embryo, and in particular the chicken embryo, has a 2000 year history in the study of animal development (Stern, 2004). The sustained use of this animal model can be attributed to a number of advantageous features including ready availability of fertilized chicken eggs, low cost and maintenance of eggs, rapid embryonic development, and easy access to the embryo within the egg for observations and experimental manipulations. Further, the chick embryo is a valid model to inform on human development since early embryonic chick and human morphology and development are very similar, and the chicken and human genomes have significant homology of approximately 60% (International Chicken Genome Sequencing, 2004). With these features in mind

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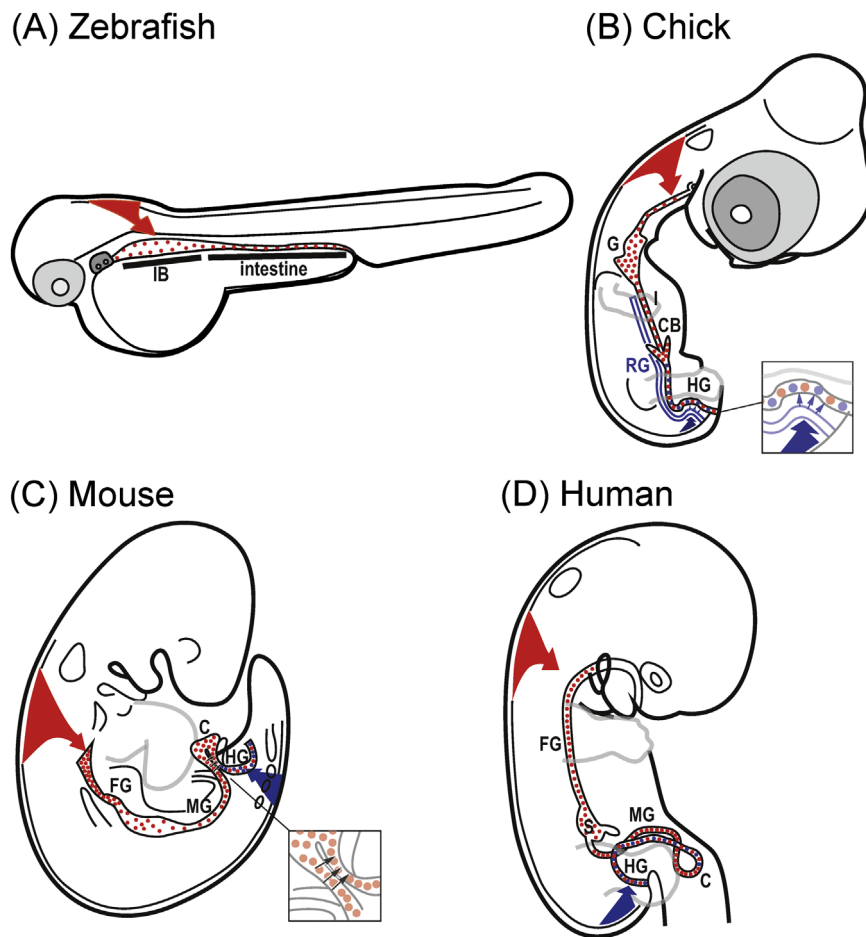


Fig. 1. Embryonic origins of the ENS in diverse vertebrate models. (A) The ENS of zebrafish derives from vagal NCC (red arrow) that enter the rostral gut tube at approximately 36 hours post-fertilization (hpf). Cells migrate caudally and progressively colonize the intestinal bulb (IB) (analogous to the stomach of mouse and human) and intestine. The gut is fully colonized by these vagal neural crest-derived ENS progenitors (red dots) by 66 hpf. As yet there is no evidence for any sacral contribution to the ENS in zebrafish. (B) In the chick, the ENS is formed primarily from vagal NCC at the level of somite 1–7 (red arrow) that enter the foregut (FG) at approximately embryonic day (E) 3–3.5 and migrate caudally to progressively colonize the gizzard (G) (mechanical stomach), intestine (I), cecal buds (CB) and hindgut, a process that is complete by E7.5 (red dots). Sacral NCC, arising caudal to somite 28 (blue arrow), also contribute to the ENS, first forming the extramural nerve of Remak (RG) (blue), and then migrating into the hindgut (inset, blue arrows) to colonize primarily the distal hindgut (blue dots). (C) The mouse ENS is formed principally from vagal NCC from the level of somite 1–7 (red arrow), which enter the foregut at approximately E9, and migrate caudally to colonize the foregut (FG), midgut (MG), cecum (C), and hindgut (HG) (red dots). In addition to rostrocaudal migration, trans-mesenteric migration of vagal NCC from the midgut to the hindgut also occurs (inset, arrows). Colonization of the length of the gut is complete by E14. An additional ENS contribution arises from NCC that migrate from the sacral region (caudal to somite 25) (blue arrow). These cells initially form pelvic ganglia adjacent to the hindgut, then migrate into the gut and primarily occupy the hindgut and caudal midgut (blue dots). (D) In the human, the ENS derives from vagal NCC (red arrow) that enter the foregut (FG) at 4 weeks of gestation and migrate along the gut to fully colonize the foregut, stomach (S), midgut (MG), cecum (C), and hindgut (HG) by week 7 (red dots). It is inferred, from mouse data, that sacral NCC also contribute to the hindgut ENS (blue hatched arrow), however no experimental evidence is yet available to confirm this. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

it is not surprising that many fundamental aspects of developmental biology such as neural crest migration and fate, limb patterning, neural tube patterning, somite segmentation, and left-right asymmetry have been elucidated using the chicken embryo (see Stern (2004)).

2.1. Early development and organization of the chick ENS

Like other vertebrates, the chicken gastrointestinal (GI) tract develops from a uniform tube-like structure where reciprocal epithelial-mesenchymal interactions pattern this organ into regions with specific morphologies and functions. Sonic hedgehog signals originating from the epithelium induce region-restricted expression of genes, such as homeotic genes (*Hox*, *Nkx*) and *Bmp4* in the mesenchyme, which in turn signals back to the epithelium to control patterning and differentiation along the anteroposterior (AP) axis (reviewed in Roberts et al. (1998)). Although grossly similar to the mammalian digestive system, the chicken GI tract has a number of key anatomical differences that are partly due to the

fact that birds do not have teeth for the breakdown of food by chewing, but instead have mechanical breakdown within the digestive system. Thus the GI tract of the chicken comprises the esophagus, crop (temporary storage pouch), proventriculus (glandular stomach), gizzard (mechanical stomach), small intestine, ceca (paired blind pouches), and large intestine (colon). Another key difference is that avians have a cloaca, an orifice that serves as the single opening for the digestive, reproductive, and urinary tracts.

The chick embryo has been used for numerous pioneering studies on ENS development (Kuntz, 1910; Le Douarin and Teillet, 1973; Yntema and Hammond, 1954). Although Yntema and Hammond first identified a vagal neural crest origin for the ENS, Le Douarin and Teillet mapped the precise location of ENS precursors within the neural crest by using the now classical quail-chick grafting technique to selectively label regions of the neural axis. These authors demonstrated that the majority of the ENS precursors along the entire GI tract originate from neural crest adjacent to somites 1–7 (Le Douarin and Teillet, 1973). This and other

studies (Burns and Le Douarin, 1998; Epstein et al., 1994; Tucker et al., 1986) showed that vagal NCC enter the pharyngeal region of the gut around embryonic day (E)3–3.5 then they migrate in a rostrocaudal direction along the growing gut to colonise its entire length by E7.5. In addition to this vagal contribution to the ENS, Le Douarin and Teillet (1973) also suggested that a second, more caudal region of the neuraxis, the sacral neural crest, caudal to the 28th pair of somites, may also contribute cells to the ENS, an idea subsequently confirmed by Burns and Le Douarin (1998). These and other studies showed that sacral NCC initially form an extramural nerve, the nerve of Remak, and from there migrate into the hindgut along this nerve's projections to contribute to the post-umbilical ENS, primarily to the distal hindgut where they comprise up to 17% of enteric neurons (Burns et al., 2000; Burns and Le Douarin, 1998; reviewed in Burns and Le Douarin (2001)).

This basic pattern of rostrocaudal gut colonization by vagal NCC, as demonstrated in the chick, appears to be similar in other vertebrate species including the zebrafish (see sections below), mouse (Wang et al., 2011; Young et al., 1998) and the human (Wallace and Burns, 2005), although there are differences in some details. For example, the development of the myenteric and submucosal plexuses in the mammalian large intestine differs from that in the avian large intestine. In the former, NCC colonise the myenteric plexus region prior to the submucosal, whereas in the latter the submucosal region is colonized before the myenteric. Also, it remains to be determined whether vagal NCC undergo so-called trans-mesenteric migration, from the midgut to the hindgut via the mesentery, in the chick and human, as has been described in the mouse (Nishiyama et al., 2012). However, this seems unlikely at least in the chick as the midgut and hindgut are not juxtaposed during gut colonization by NCC as they are in the mouse.

Although the sacral contribution to the hindgut has been relatively well defined in the chick, the main entry route for sacral NCC into the hindgut appears to be along nerve fibers that originate in the nerve of Remak. However, this extramural nerve is unique to avians, raising questions about the contribution of this population of NCC to the gut in other amniotes. Nevertheless, sacral NCC form extramural pelvic ganglia in mammals and studies in mice have shown that, similar to the chick, sacral-derived NCC migrate along nerve fibers that extend from pelvic ganglia into the proximal hindgut where they contribute neurons and glial cells to the hindgut ENS (Wang et al., 2011). However, sacral NCC do not appear to contribute to the ENS in the zebrafish (see below) and the sacral contribution to the human ENS has not been directly analysed due to the technical limitations of labeling live cells in human tissues.

2.2. Conserved molecular mechanisms of chick ENS development

Arguably the most important signaling pathway in ENS development is the RET pathway, and a number of important studies in mice have shown that loss of the Ret receptor, its co-receptor Gfra1, or its ligand Gdnf, results in the failure of NCC to colonise the gut and form the ENS (reviewed in Sasselli et al. (2012)). Studies in chick have shown that Ret and Gfra1 are also expressed by vagal NCC, and that Gdnf is expressed in the gut (Burns and Delalande, 2005). Using *in situ* hybridisation, immunohistochemistry and DNA microarrays, Delalande et al. (2008) also demonstrated the expression of established ENS regulators *Ret*, *Mash1*, *Sox10*, *Pax3*, *Phox2b*, *Grfα1*, *Gfra2*, *Ednrb*, *Ece-1*, *NT-3* and *TrkC* in vagal and sacral NCC of the chick, highlighting the conservation of molecular mechanisms between the chick and the mouse. This conservation holds true for other key pathways in ENS development including the Hedgehog (Nagy et al., 2016), Bmp (De Santa Barbara et al., 2005; Goldstein et al., 2005), and Notch (Faure et al., 2015)

pathways, as well as for extracellular matrix components, the expression of which are conserved in the gut across species (Nagy et al., 2012) and further supports the use of the chick embryo for studies involving candidate ENS genes and signaling pathways.

2.3. Unique molecular tools available for chicken embryos

2.3.1. Transgenic GFP chickens for selective cell labeling

In 2004, McGrew et al. (2004) used lentiviral vectors to generate transgenic chickens that ubiquitously express green fluorescent protein (GFP) in all cells. These GFP expressing chickens are maintained, and fertilized eggs made available to the research community, at the Transgenic Chicken Facility which is part of the National Avian Resource Facility (UK) <http://www.narf.ac.uk/transgenic/>. These GFP-expressing tissues can be used in a similar manner to the classic quail-chick grafting technique, by microsurgically grafting GFP tissues into wild type chick embryos to permanently mark and fate-map NCC. For example, using such an approach, Freem et al., found that transplanted GFP+ neural tube integrated into the host chick embryo and GFP+ NCC migrated extensively along stereotypical pathways to colonise the gut and primitive lungs (Freem et al., 2012). Delalande et al., also recently used the chick^{GFP}-chick technique in combination with vital carbocyanine dye (DiI) labeling of blood vessels to investigate interactions between migrating vagal NCC and developing blood vessels within the GI tract (Delalande et al., 2014, 2015). The use of GFP+ chick tissues for labeling NCC has a number of advantages. Since GFP expression in the transgenic chick line is cytoplasmic, cell bodies and their projections can be observed, allowing visualisation of intricate neuronal networks that are not apparent when using quail-chick grafting, where only the quail cell nucleus is marked with the quail cell-specific perinuclear antigen (QCPN) antibody. In addition, the GFP fluorescence is extremely bright, and GFP+ cells are easily discernible in live recipients even without immunostaining for GFP. This allows pre-screening of successful grafts *in ovo* using fluorescence microscopy, whereas with quail-chick grafting the embryo must be killed, fixed and immunohistochemically stained before the success of the graft can be ascertained. Thus not only is chick^{GFP}-chick intraspecies grafting a significant technical advance allowing efficient and reliable tracing of NCC migration and development, but it also opens the door to further *in vivo* chick studies and other techniques useful for studying ENS developmental biology including cell sorting based on GFP fluorescence, organotypic culture of tissues containing GFP+ cells, and timelapse imaging of GFP+ cell migration within the gut. Interestingly, due to the success of the transgenic GFP chicken line for developmental biology studies, other transgenic chicken lines may soon be available. These include a Cre-lox system with inducible expression of tdTomato either by Cre protein or injection of a Cre-expressing plasmid, a membrane GFP line, and a Notch reporter where Venus (yellow) is expressed under the Hes5-1 promoter when Notch signaling is active (see <http://www.narf.ac.uk/transgenic/> for further information and availability).

2.3.2. Mutant chickens with gut neuromuscular defects

Over the years, a number of chicken mutants have served as important experimental models for human conditions (Delany, 2004). Perhaps most relevant for this current article are the *talpid* mutants which have limb defects and are so called because their paddle-shaped limbs resemble the limbs of the mole (*Talpa*). Three distinct, naturally occurring, autosomal recessive, lethal *talpid* mutations (*talpid*, *talpid*² and *talpid*³) were originally described (Abbott and Baskin, 1960; Cole, 1942; Ede and Kelly, 1964). The *talpid* mutant is now extinct, but *talpid2* and *talpid3* flocks maintained at the College of Agricultural and Environmental Sciences,

University of California Davis, USA and The Transgenic Facility, The Roslin Institute, UK respectively, have been used to study a number of developmental processes. For example, the *talpid3* mutant, in addition to limb defects, has malformations including face, skeleton, and vascular defects which are now known to involve defective Hedgehog signaling (Davey et al., 2006). The *talpid3* gene, initially identified as the uncharacterized gene *KIAA0586*, was subsequently found to encode a centrosomal protein essential for primary cilia formation, the site where hedgehog signal transduction occurs (Yin et al., 2009), thus strengthening the link between the phenotypic defects and hedgehog signaling. More recently, *talpid2*, which has a 19 bp deletion in exon 32 within *C2CD3* (*C2* calcium-dependent domain containing 3), has also been shown to affect ciliogenesis, hedgehog signaling and craniofacial development (Chang et al., 2014). In addition to the defects mentioned above, *talpid3* chick embryos have a number of defects in GI tract development. These include decreased gut length, failure in tracheo-oesophageal separation, open hindgut, and smooth muscle and ENS patterning defects (Burns et al., 2009). Thus *talpid3* provides an accessible chicken model to study the role of hedgehog signaling in gut and enteric neuromuscular development.

2.3.3. Alteration of gene function during embryonic chick development

One of the limitations in the use of the chicken embryo during the “molecular era” of developmental biology in the 1980–1990s was the lack of genetic approaches to modulate gene function, notwithstanding the use of recombinant retrovirus and adenovirus for gene transfer and cell lineage analysis (Frank and Sanes, 1991; Yamagata et al., 1994). However, with the advent of *in ovo* electroporation, ectopic gene expression or overexpression analyses were made possible (reviewed in Funahashi and Nakamura (2008), Itasaki et al. (1999), and Nakamura and Funahashi (2013)). Subsequent studies using expression vectors, siRNA constructs, and tissue specific reporters demonstrated that such approaches can also be used for loss-of-function analyses, including knockdown experiments with morpholinos and RNAi allowing efficient alteration of gene function during embryonic development (Das et al., 2006; Sauka-Spengler and Barembaum, 2008). Both these overexpression and gene silencing approaches have been used to elucidate the roles of various genes, including Ret, in ENS development (Delalande et al., 2008; Mwiszerwa et al., 2011). The more recent development of technologies such as transcription activator-like effector nuclease (TALEN) and the CRISPR/Cas9 system, promise to revolutionize genetics research by allowing genome editing in different organisms. Proof of concept studies using these approaches have already been performed in the chick embryo *in vivo*: TALENs has been used successfully to generate specific gene knockout (Park et al., 2014) and loss of function studies have been performed using gene electroporation and CRISPR/Cas9 to target the transcription factor PAX7 in tissues of the developing chick embryo (Veron et al., 2015).

The chick embryo has also been used in studies investigating the regulatory mechanisms of gene expression. For example, studies by Zhu et al. (2014), including *in ovo* electroporation in chick embryos, investigated the regulatory mechanisms of HOXB5 on RET transcription to obtain a better understanding of the etiology of Hirschsprung disease (HSCR). Similar studies, investigating the gene regulatory elements for NCC-specific expression of various transcription factors, have been carried out in the chick by the Bronner group (Barembaum and Bronner, 2013; Betancur et al., 2010).

Such previous and ongoing gene manipulation studies highlight the potential for investigating the roles of genes involved in ENS development in the chick. When considered along with

chicken, mouse and human genome data, these approaches will help to elucidate the function of chicken genes and their orthologs in other species.

2.3.4. The chicken CAM as a platform for ENS development, cell replacement therapy and gut tissue engineering studies

The highly vascularised chorioallantoic membrane (CAM) of the chicken egg comprises the outermost extraembryonic membrane and is essential for chick development. The CAM begins to form at E5–6 and progressively extends, adhering to the acellular inner shell membrane, until it surrounds the embryo and other egg contents by E11–12. Over the last 30–40 years, the CAM has become a well-established and effective platform for studying a number of biological processes such as toxicity, gas exchange, drug delivery, tumor invasion and growth, and angiogenesis (reviewed in Deryugina and Quigley (2008), Lokman et al. (2012), and Ribatti (2012)). For decades the CAM has also been used for studies of ENS development (Newgreen et al., 1980). Various combinations of tissues (e.g. different neural crest regions) can be combined with different gut regions (usually “aneural” midgut or hindgut regions prior to the arrival or vagal NCC) and cultured together on the CAM to assess the ENS developmental potential of NCC (Hearn and Newgreen, 2000; Zhang et al., 2010). The culture of aneural chick hindgut on the CAM was also successfully used to confirm that interstitial cells of Cajal (ICC) are mesenchyme-derived cells since c-kit+ ICC were found to develop in CAM-cultured gut that was devoid of ENS cells (Lecoin et al., 1996). An alternative to the CAM for *ex vivo* tissue combinations is the chicken coelomic cavity, where aneural hindgut, isolated from avians or rodents prior to the arrival of neural crest cells, is transplanted into the coelomic cavity of a host chick embryo (reviewed in Nagy and Goldstein (2006)). Here NCC from the host chick can colonise the gut explant, and when combined with analysis by immunohistochemistry or vital dye labeling the origin of the host-derived cells, their migration patterns, their capacity to differentiate, or their ability to influence their surrounding gut micro-environment (Akbareian et al., 2013) can be ascertained.

More recently, the CAM has been used in the rapidly growing field of tissue engineering and regenerative medicine by facilitating rapid, simple, low-cost screening of blood vessel interactions with biomaterials/tissues placed on its surface (Bauguera et al., 2010; Maghsoudlou et al., 2013; Totonelli et al., 2012). Further, due to the remarkable ability of the CAM to support, by neovascularisation, the growth and maintenance of live tissues, the CAM has been used as an “*ex-vivo*” system for testing the ability of ENS stem cells to colonise embryonic gut grown and maintained on the CAM (Metzger et al., 2009). Ongoing studies in a number of labs are now using the CAM as a “medium throughput” system to assess the ability of stem cells from various sources to colonise gut (including aganglionic gut) grown on its surface, prior to moving to *in vivo* studies in animals.

3. The zebrafish as a model for ENS development

In comparison with the avian and murine model systems the zebrafish model is a relative newcomer to the field of ENS development. Zebrafish was developed as a new model organism in the 1980s, when search for a simple vertebrate organism that could be studied using both embryological and genetic methods lead to *Danio rerio* (Streisinger et al., 1981). The ability to obtain large numbers of external fertilized and synchronously developing transparent embryos made zebrafish an attractive model that was quickly adopted by large numbers of labs, and its use continues to expand. Sequencing of the zebrafish genome shows that 70% of human genes are related to genes found in zebrafish and that

~85% of disease associated human genes have a zebrafish orthologue (Howe et al., 2013), making this a highly relevant model for studying ENS development and for modeling human diseases.

3.1. Early development and organization of the zebrafish ENS

As with other vertebrate systems the zebrafish GI tract is a complex organ comprised of multiple cell types including epithelial, muscle, vascular, immune and neuronal cells. The teleost gut is similar to that in amniotes although its overall structure is less complex (Wallace et al., 2005). For example the zebrafish GI tract does not have a distinct stomach though there is an enlarged region of the anterior intestine, known as the intestinal bulb. This region displays patterns of motility as well as goblet cells that produce acid and neutral mucins like the mammalian stomach (Holmberg et al., 2007; Ng et al., 2005). Furthermore gene expression patterns of *sox2*, *barx1*, *gata5* and *gata6* also resemble those of the developing mammalian stomach (Muncan et al., 2007). The zebrafish gut epithelium is also simpler than that of amniotes in that it lacks crypts and is arranged in broad irregular folds rather than forming villi (Ng et al., 2005; Wallace et al., 2005). Zebrafish also lack a submucosal layer with the vascular tissue in the intestine occurring in the mucosa and the muscularis (Wallace et al., 2005).

The zebrafish ENS, like that of all other vertebrates, is derived from the neural crest (Kelsh and Eisen, 2000). A potential difference though is that unlike amniotes, where both vagal and sacral crest contribute to the ENS, the zebrafish ENS is derived from the vagal crest, with no evidence for sacral neural crest contribution (Elworthy et al., 2005; Furness, 2006; Olden et al., 2008; Shepherd et al., 2004). The migration of the zebrafish enteric neural crest cells (ENCCs) along the gut also appears simpler than in amniotes. Instead of migrating as multiple chains that have complex unpredictable trajectories, zebrafish ENCCs migrate as two parallel chains along the length of the developing gut (Elworthy et al., 2005; Olden et al., 2008; Shepherd et al., 2004). Subsequently ENS precursors migrate circumferentially around the gut and differentiate into enteric neurons and glia. The final organization of the zebrafish ENS is again simpler than that of amniotes in that there is only a myenteric plexus that is composed of single neurons or small groups of neurons rather than more complex ganglionated myenteric and submucosal plexuses. Differentiation of ENCCs occurs in a rostral to caudal progression, with first neuronal differentiation markers observed in rostral regions at around 2.25 dpf and by around 3 dpf differentiated ENS neurons are seen along the full length of the gut (Olden et al., 2008). The gut is fully functional at 7 dpf (Holmberg et al., 2007), and at this point the intestinal ENS is comprised of several hundred neurons. Currently, evidence for the presence of glial cells in the zebrafish ENS is limited and partially conflicting (Doodnath et al., 2010; Germana et al., 2008; Hagstrom and Olsson, 2010), with some reports observing glial cell markers in the zebrafish gut (i.e. GFAP), and others observing a lack of glial cell markers (i.e. S100). Clearly more information is needed about this important cell type.

3.2. Enteric neuronal subtype differentiation in zebrafish

A common feature of the ENS in all vertebrate species is the presence of numerous neuronal subtypes including motor neurons, interneurons, and intrinsic primary afferent (sensory) neurons. Each of these classes can be further subdivided based on neuronal morphology, physiology, and biochemistry. Extensive immunohistochemical analysis in amniotes has revealed that each ENS neuron expresses several different neurotransmitters, and the chemical coding hypothesis proposes that the combinatorial expression of these neurotransmitters can be used to functionally

define each neuronal class (Furness, 2006). Studies from a number of different laboratories indicate that, similar to amniotes, both excitatory and inhibitory neurotransmitters are expressed by neurons in the zebrafish ENS. Thus, enteric neurons expressing adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), calcitonin gene-related polypeptide (CGRP), neuronal nitric oxide synthase (nNOS) neurokinin-A (NKA), substance P, acetylcholine and serotonin have all been reported (Holmberg et al., 2006, 2004; Holmqvist et al., 2004; Kuhlman and Eisen, 2007; Olden et al., 2008; Olsson et al., 2008; Pietsch et al., 2006; Poon et al., 2003). The precise chemical coding of the embryonic, larval, and adult zebrafish ENS and a detailed description of the morphologies of the different types of zebrafish enteric neurons does not yet exist. However the chemical coding in the zebrafish ENS has been addressed (Uytenbroek et al., 2010, 2013). The conclusion from these studies is that overall the distribution and timing of the appearance of different neuronal markers in the developing zebrafish ENS is comparable to that seen in amniotes.

3.3. Conserved molecular mechanisms of zebrafish ENS development

Despite developmental and structural differences between the ENS of zebrafish and amniotes, most of the molecular mechanisms underlying ENS development are conserved among species. For example, signaling through the RET receptor tyrosine kinase is critical for ENS development in both amniotes and zebrafish. *ret* mRNA is expressed in ENS precursors and differentiating neurons in the developing zebrafish gut (Bisgrove et al., 1997; Marcos-Gutierrez et al., 1997; Shepherd et al., 2004). An evolutionarily conserved role for *ret* is demonstrated in morpholino gene knockdown experiments, where loss of *ret* leads to absence of ENS neurons along the length of the gut (Shepherd et al., 2004). As in other vertebrate species, zebrafish have two *ret* isoforms (*ret9* and *ret51*), of which *ret51* is unnecessary for complete colonization of the gut by ENS precursors (Heanue and Pachnis, 2008). RET acts together with GFR α , a member of the family of GPI-anchored cell surface receptors, to form a receptor complex that mediates signals of the GDNF neurotrophic factor family (Airaksinen and Saarma, 2002). Zebrafish has two GFR α 1 orthologs and morpholino-mediated antisense knockdown of the two orthologs (GFR α 1a and GFR α 1b) results in complete loss of ENS neurons and their precursors (Shepherd et al., 2004). Similar to knockdown of RET and GFR α 1, knockdown of GDNF also results in complete loss of zebrafish ENS neurons and their precursors (Shepherd et al., 2001). Two other GDNF family members, Neurturin and Artemin, are reported, by immunoreactivity, to be present in zebrafish, although their function in zebrafish ENS development is unknown (Lucini et al., 2005, 2004). Interestingly, the role of the Endothelin signaling pathway, which interacts with the RET signaling pathway during development of the ENS in mammals and avians (Landman et al., 2007), is potentially not required for zebrafish ENS development, as a mutation that perturbs the function of the Endothelin receptor, *Ednr1a*, does not result in an ENS phenotype in zebrafish (Parichy et al., 2000). However a second Endothelin receptor type B ortholog, *ednr1b*, has recently been identified and it appears to be required for ENS development in zebrafish. Like Endothelin receptor type B there are two zebrafish Endothelin 3 orthologs (Braasch et al., 2009). Which ortholog is required for ENS development is unknown, though morpholino knockdown of the *edn3b* showed that it was required for iridophore pigment cell development in zebrafish (Krauss et al., 2014).

In addition to RET signaling, the function of several transcription factors identified in mouse and avian as potential regulators of *Ret* expression has also been tested in zebrafish ENS development (Burzynski et al., 2009). Mutants lacking Sox10 (Dutton et al.,

2001; Kelsh and Eisen, 2000) or *Foxd3* (Montero-Balaguer et al., 2006; Stewart et al., 2006) lack an ENS, as do zebrafish in which *Phox2b* (Elworthy et al., 2005) or *Pax3* (Minchin and Hughes, 2008) have been knocked down with morpholino antisense oligonucleotides.

Furthermore like the GDNF signaling pathway the Hedgehog signaling pathway also appears to have an evolutionarily conserved role in zebrafish ENS development. Zebrafish mutants for *shh* or *ihh* have ENS defects similar to those seen in respective mouse knockouts (Korz et al., 2011; Reichenbach et al., 2008).

3.4. Unique molecular tools available for zebrafish

3.4.1. Live imaging of cell behavior in zebrafish

The ENS derives from a population of NCCs that undertake an extended migratory journey to reach their target within the lengthening gut. To understand how this achieved, it is necessary to visualize the process, a feat not possible *in vivo* using murine models due to the constraints of internal development. One of the obvious benefits of studying developmental processes in zebrafish is the accessibility of embryos from fertilization onwards. Moreover because embryos are optically translucent (albino larvae or those chemically treated with PTU remain translucent into later larval stages), fluorescently labeled cell populations can be detected and tracked throughout their development, even deep within the embryo. This feature has been exemplified in beautiful studies of mass cell movements during development and also for high-resolution analysis of individual cells within populations, such as the posterior-lateral line (reviewed in Clarke (2009)).

Within the developing ENS, these same methodologies have been used to live-image ENS colonization of the gut for the first time in any vertebrate system. Using a transgenic line where a zebrafish *phox2b* ENS specific enhancer (McGaughey et al., 2009, 2008) drives expression of the fluorescent protein Kaede, time-lapse movies tracked the ENS lineage along the developing gut (Harrison et al., 2014). Such experiments show that the ENS colonises the gut at a constant rate during development. Kaede is a photoconvertible protein; normally Kaede fluoresces green, but upon UV light exposure, the protein changes to expressing red fluorescence (Ando et al., 2002; Mizuno et al., 2003). Therefore, in zebrafish, it is possible to target UV light to specific Kaede expressing cells at any time or place during migration thus allowing the behavior (including migration, proliferation, cell death and differentiation) of groups of cells, or even individual cells, to be monitored. Similar approaches have been used in mouse to track the migration of cells through the explanted colon (Young et al., 2014), revealing surprisingly dynamic and directionally variable migratory behaviors. The zebrafish system has the advantage of allowing cells to be traced *in vivo* even from the earliest stages of gut colonization. Furthermore, because zebrafish can be visualized for several days, they can be monitored from their migration into and along the gut through to their differentiation (when ENS neurons number only in the hundreds of cells – a highly tractable number). Therefore, it is possible to label an individual cell, track and label its progeny, and analyse (post-hoc) to determine which differentiated lineages arise. Such analysis could be used to address important questions such as the lineage relationships between cells of different neuronal subpopulations.

3.4.2. Gene functional studies

Zebrafish were developed as a tool for studies of vertebrate development in part because of the ability to perform forward genetic screens to identify new genetic regulators of biological processes (Driever et al., 1996; Haffter et al., 1996). In addition to their optical accessibility, zebrafish are easy to maintain and breed. Although their generation time is not especially short (~3

months), embryos develop rapidly and can be generated in large numbers, features that facilitate collection of mutants and genetic mapping (Westerfield, 2007). From the earliest screens, interesting mutants were identified affecting the neural crest (Kelsh et al., 1996; Kelsh and Eisen, 2000), and continued screening identified further mutants affecting the ENS (Kuhlman and Eisen, 2007; Pietsch et al., 2006). Positional cloning identified the genes mutated in some cases, including the transcription factor *sox10* (Dutton et al., 2001), (also a known ENS regulator in mouse (Herbarth et al., 1998; Southard-Smith et al., 1998) and the previously undescribed *med24* (Pietsch et al., 2006). The remaining mutations, which could be either gene coding mutations or gene regulatory mutations in known or novel ENS genes, remain to be identified.

In addition to the capacity to perform these unbiased screens, the ability to use a reverse genetic approach to rapidly generate gene 'knock-downs' has been a long-recognized strength of working with zebrafish. A morning's work of injecting antisense morpholino oligos (MOs), targeted against a gene of interest, into one-cell stage embryos can generate hundreds of putative knock-down embryos for analysis (Bill et al., 2009). This strategy was used to study the roles of *gdnf*, *gfr α 1*, *ret*, *phox2b*, and demonstrated that gene function of each of these genes was required for normal ENS development (Elworthy et al., 2005; Heanue and Pachnis, 2008; Shepherd et al., 2001, 2004). More recently, genome editing approaches, such as zinc finger nucleases, TALEN and now CRISPR/Cas9, are enabling the efficient and rapid generation of targeted mutations in genes of interest (Peng et al., 2014), and are likely to play a major role in future studies.

The ease of knock-down strategies is being exploited to a great extent by clinical groups to validate candidate HSCR susceptibility loci. Genome-wide association studies (GWAS) are identifying candidate HSCR susceptibility loci (reviewed in Alves et al. (2013)), and MO studies in zebrafish are now being used to screen for genes required for normal ENS development. For example, GWAS identified a non-coding variant within the class 3 Semaphorin gene cluster, and a role for *sema3* was tested by MO knock-down (Jiang et al., 2015). These studies showed that *sema3* knock-down embryos had a reduction in ENS neurons. Moreover, an interaction between *sema3* and *ret* could be demonstrated, since when *sema3* knock-down is combined with *ret* knock-down, ENS neurons are dramatically reduced in number (Jiang et al., 2015). As human genetic studies increase in their power, the number of candidate genes to screen for functional requirement will increase. It is likely that zebrafish will continue to play a significant role in the pipeline in these studies. One issue that must be contended with, however, is the fact that human genes often have more than one orthologous gene in zebrafish (Howe et al., 2013), potentially requiring both zebrafish genes to be targeted to replicate mutation of a single human gene (i.e. Shepherd et al. (2004)).

The ability to generate transgenics has been an additional resource for study of HSCR disease. Human genetics studies have highlighted that to understand HSCR disease susceptibility, it is crucial to also consider the influence of non-coding mutations. For example a clear association of HSCR susceptibility has been made to a common non-coding mutation in intron 1 RET (Emison et al., 2005). Zebrafish has demonstrated potential to identify and study gene regulatory regions of interest. For this analysis, transgenic zebrafish are generated to test the ability of non-coding regions (of zebrafish, mouse or human origin) to drive reporter expression in appropriate patterns. Although transgenesis has a similar efficiency in mouse and zebrafish, the sheer number of transgenics that can be generated in a single session, makes zebrafish a useful model for these studies. Such analysis has been used to identify key regulatory/functional domains in *RET*, *Sox10/SOX10*, and *phox2b* (Antonellis et al., 2008; Fisher et al., 2006; McGaughey

et al., 2008). Critically, this approach provides a method to identify important regulatory regions which would otherwise be missed if using simple sequence conservation as an indicator.

Screens for non-genetic regulators of ENS development are also employing zebrafish as a model to understand dietary and environmental factors affecting ENS development. By simply rearing embryos in water containing compounds of interest, the influence on ENS development can be tested. Such screens in zebrafish identified mycophenolic acid (MPA), a commonly used immunosuppressant, and ibuprofen, as inhibitors of ENCC migration (Lake et al., 2013; Merrick Schill et al., 2015). Future work, combining studies of genetic and non-genetic regulators of ENS development in zebrafish, is likely to provide valuable insight into HSCR pathophysiology.

3.4.3. *In vivo gut motility studies*

The optically translucent zebrafish provides unmatched potential for *in vivo* imaging of gut motility amongst model systems. Zebrafish embryos subsist on a yolk store until 5 dpf, when both the mouth and anal pore are open and larvae begin to feed (Wallace and Pack, 2003). Because the gut is a relatively simple tube, lying ventrally just over the depleting yolk sac, it is possible to image the gut motility waves as they move along the gut wall using a standard dissecting microscope. While contractions of the gut wall can be observed from 4 dpf, organized motility patterns are detected at 7 dpf, with distinct motility behaviors observed in different regions (Holmberg et al., 2003). In the intestine, antegrade waves (anally propagating) are the major feature, and within the intestinal bulb, which is analogous to the stomach of terrestrial vertebrates, retrograde (orally propagating) waves predominate (Holmberg et al., 2003; Kuhlman and Eisen, 2007; Rich et al., 2013). Recordings of gut motility can be converted into spatio-temporal maps (STMs), allowing details of the motility patterns to be represented, and key information to be extracted (Holmberg et al., 2007). Using such assay systems, relatively simple *in vivo* experiments can be conducted, such as those showing that the sodium-channel blocker Tetrodotoxin (TTX) disrupts organized motility patterns, highlighting the important role that the ENS has on regulating gut motility (Holmberg et al., 2007). Another simple assay that can be used to assess gut motility involves using a fluorescent tracer (larval feed mixed with fluorescent beads), and recording time needed to transit the ingested material (Field et al., 2009). This method has been used to demonstrate impaired intestinal transit in zebrafish lacking ENS neurons (Abrams et al., 2012; Field et al., 2009).

The ability to easily assess functional consequences of ENS loss has been used to explore mutants initially identified in forward-genetic screens (Kuhlman and Eisen, 2007). *colorless* (*sox10*) mutants, which lack ENS neurons in the gut (Dutton et al., 2001; Kelsh and Eisen, 2000), showed a loss of organized motility patterns (Kuhlman and Eisen, 2007). Mutants that have reduced numbers of ENS neurons, such as *gutwrencher*, also exhibit uncoordinated contractions, with multiple discrete regions contracting simultaneously (Kuhlman and Eisen, 2007). These experiments reveal a correlation between ENS neuron number and organized gut motility. In future it will be of interest to explore the role of specific cell types in controlling motility patterns using specific neuromodulators (i.e. Holmberg et al. (2006, 2004)). The ability to correlate cell loss with gut motility outcomes is likely to play an increasingly important role in the emerging screens for HSCR modifier loci (see above). With a simple set of experiments, another layer of information can deepen our understanding of roles played by these loci in ENS development.

The ENS interacts with mesoderm-derived interstitial cells of Cajal (ICCs) and smooth muscle cells to elicit coordinated motility behaviors in the smooth muscle of the gut wall (Furness, 2006;

Uyttbroek et al., 2013). However, these distinct systems are often studied in isolation. For example, independent experiments demonstrate the roles in regulating intestinal motility for the ENS (Kuhlman and Eisen, 2007), for smooth muscle (Davuluri et al., 2010), and for ICCs (Rich et al., 2013). The relative simplicity of the zebrafish gut, in terms of a tractable number of ENS neurons and ICCs and defined motility behaviors, lends itself to exploring the interactions of these cell populations in greater detail. Some such studies have suggested that at early stages, smooth muscle motility operates independently of other systems, since motility occurs in the absence of the ENS, whereas later the ENS is required for organized intestinal motility (Abrams et al., 2012; Davuluri et al., 2010).

A research area where the zebrafish model has great, untapped potential to progress ENS studies is in dissecting the neural circuits regulating motility. Because mature gut motility patterns are observed when the gut tube is a simple, linear structure with only hundreds of ENS neurons, the possibility exists to correlate *in vivo* the activity of neurons or groups of neurons with local functional output (muscular contractions). From studies in the central nervous system, a wide range of tools are available to give a read-out of neural activity; transgenic lines exist that encode calcium indicators, such as GCaMPs (Muto et al., 2013), or allow permanent marking of neurons active at a particular time (Fosque et al., 2015). Such tools can be used to establish a map of neural activity in relation to output and provide a basis to understand ENS circuitry. In addition, new neuromodulator transgenic zebrafish, such as those outlined for study of prey capture behavior (Muto and Kawakami, 2013), would allow specific neurons to be stimulated or inhibited. These, and other emerging tools, can be used to understand neurofunctional units and their control. This information will be increasingly important for the application of cell replacement therapies for HSCR (see article by Burns et al. 2016).

4. Summary

Here we highlighted how the chick embryo has been well established as a classical model for the study of ENS development for a considerable time. However, recent advances such as the alteration of gene function and the development of transgenic chicken lines, as well as “repurposing” more established techniques such as the chick CAM, will help to ensure that the chick embryo remains at the forefront of developmental biology studies for many years to come. Similarly, the biological features and technical approaches unique to the zebrafish perfectly place this model to unravel the genetic mechanisms underlying normal and abnormal development of the ENS, and to study ENS control of gut motility.

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