Int. J. Dev. Biol. 68: 93-102 (2024)
https://doi.org/10.1387/ijdb.240057ar

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Origin and development of interstitial cells of Cajal

TARA SWEET[1], CHRISTEEN M. ABRAHAM[2], ADAM RICH*[,3]

[1]Departments of Biology and Psychology, SUNY Geneseo, NY, USA, [2]St. George's University School of Medicine, West Indies, Grenada, [3]Department of Biology, SUNY Brockport, 350 New Campus Drive, Brockport, NY, USA

ABSTRACT The digestive tract is a series of organs with specific functions and specialized anatomy. Each organ is organized similarly with concentric layers of epithelial, connective, smooth muscle, and neural tissues. Interstitial cells of Cajal (ICC) are distributed in smooth muscle layers and contribute to the organization of repetitive and rhythmic smooth muscle contractions. Understanding ICC development is critical to understanding gastrointestinal motility patterns. Experiments determining ICC origin and development in mice, chicken, and humans are described, as well as what is known in the zebrafish. At least six types of ICC in the digestive tract have been described and ICC heterogeneity in adult tissues is reviewed. Factors required for ICC development and for maintenance of ICC subclasses are described. This review is suitable for those new to ICC development and physiology, especially those focused on using zebrafish and other model systems.

KEYWORDS: interstitial cell of Cajal, ICC, zebrafish, development, gastrointestinal motility

Introduction

The smooth muscle layers of the digestive tract are complex and composed of many cell types, including muscle cells, nerve cells, mast cells, macrophage, fibroblasts, PDGFα cells and interstitial cells of Cajal (ICC), each with their own origin, development, and function. Development of the vertebrate digestive tract is generally conserved across species. Here we give an overview, highlighting distinctions between amniotes (such as humans, mice, and chicks) and anamniotes (such as zebrafish) as necessary for interpreting experiments. We also refer readers to an excellent description of the development of the zebrafish digestive tract in a recent review considering zebrafish as a model organism to study gut microbe interactions (Flores *et al.*, 2020). This article reviews the current understanding of the origin and development of ICC and evaluates the potential for future examination in zebrafish.

Initial and early digestive tract development

The digestive tract is derived from two primitive layers, the endoderm (which gives rise to the epithelium) and the mesoderm (which develops into the mesenchyme, smooth muscle layers, and numerous other cell types). Initial development of the digestive tract follows this basic sequence of events: gastrulation, formation of the primitive gut from the endoderm and positioning of the inner leaflet of the lateral plate mesoderm (splanchnic) against the endoderm (d3 in chick; E9.5 in mouse; week 4 in humans; and 34-52 hours post fertilization (hpf) in zebrafish) (Bardot and Hadjantonakis, 2020; Hamburger and Hamilton, 1992; Huycke and Tabin, 2018; Kimmel *et al.*, 1995; Spence *et al.*, 2011; Tyser *et al.*, 2021; Wallace *et al.*, 2005; Walton *et al.*, 2016). Rapid growth and folding of the embryo cause the inner leaflet of the lateral plate mesoderm to encircle the gut and become the visceral mesoderm (Chin *et al.*, 2017; McLin *et al.*, 2009; Prummel *et al.*, 2019; Prummel *et al.*, 2020). The endoderm and visceral mesoderm subsequently undergo a period of rapid growth, characterized by increased intestinal length, circumference, and luminal area (from d5-d8 in chick; from E9.5-E13.5 in mice; approximately 3-7 weeks in humans; from 34-76 hpf in zebrafish) (Cervantes *et al.*, 2009; Chin *et al.*, 2017; Huycke and Tabin, 2018; Lepourcelet *et al.*, 2005; Polak-Charcon *et al.*, 1980; Spence *et al.*, 2011; Wallace *et al.*, 2005).

During early development the mesenchyme of the gut tube begins to differentiate into multiple layers of orthogonally oriented smooth muscle (beginning at d6 in chick; E11 in mice; week 5 in humans; 50 hpf in zebrafish) (Chevalier *et al.*, 2021b; Chin *et al.*, 2017; Fu *et al.*, 2004; Gabella, 2002; Huycke *et al.*, 2019; McHugh, 1995; McKeown *et al.*, 2001; McLin *et al.*, 2009; Seiler *et al.*, 2010; Shyer *et al.*, 2013; Wallace and Burns, 2005; Wallace *et al.*, 2005)

Abbreviations used in this paper: ANO1, anoctamin 1; GI, gastrointestinal; Hpf, hours post fertilization; ICC-DMP, interstitial cells of Cajal – deep muscular plexus; ICC-IM, interstitial cells of Cajal - intramuscular; ICC-MY, interstitial cells of Cajal – myenteric plexus; ICC-SM, interstitial cells of Cajal – submucosal plexus; LRIG1, immunoglobulin-like domains protein 1; NPR, natriuretic peptide receptor; QCPN, quail non-chick peri-nuclear; SCF, stem cell factor, steel factor; VENT, ventrally emigrating neural tube.

***Address correspondence to:** Adam Rich. Lennon Hall B18, Brockport, NY 14420, USA. E-mail: arich@brockport.edu | https://orcid.org/0000-0001-7588-2632

Submitted: 21 March, 2024; Accepted: 20 June, 2024; Published online: 12 August, 2024.

ISSN: Online 1696-3547, Print 0214-6282

Printed in Spain

In amniotes, these layers include the muscularis propria and the muscularis mucosae. The muscularis propria is located medially between the submucosa and serosa and functions primarily in gastrointestinal mixing and peristalsis. It is divided into two distinct layers, an inner circular layer and an outer longitudinal layer. In humans, the stomach contains a third, middle oblique smooth muscle layer. The muscularis mucosae is located closest to the lumen, between the submucosa and lamina propria and functions to move the mucosa, which is important in both gastrointestinal mixing and absorption (McHugh, 1995; McHugh, 1996). In anamniotes, the intestinal architecture of smooth muscle is less complex and the muscularis mucosae is absent (Wallace and Pack, 2003).

Concurrent with muscle development neural crest cells migrating from the dorsal CNS populate the developing intestine (beginning at d2.5 in chick; E9.5 in mice; 4 weeks in human; 32 hpf in zebrafish (Nagy and Goldstein, 2017; Wallace and Pack, 2003; Young *et al.*, 1999). These cells give rise to the enteric nervous system (Espinosa-Medina *et al.*, 2017). This population of cells actively migrates ventrally to the most anterior region of the developing gut tube first followed by rostral to caudal development along the foregut tube in response to signals from the surrounding mesenchyme (Taraviras and Pachnis, 1999; Young *et al.*, 1999). A second population of neural crest cells migrate from the sacral region to seed the distal part of the enteric nervous system in the digestive tract of amniotes (Burns and Douarin, 1998; Gershon *et al.*, 1993; Orts Llorca, 1934; Shepherd and Eisen, 2011; Wallace and Burns, 2005; Wang *et al.*, 2011). This differs in zebrafish where the enteric nervous system is derived entirely from the vagal neural crest (Dutton *et al.*, 2001; Kelsh and Eisen, 2000; Rocha *et al.*, 2020; Sasselli *et al.*, 2012).

During this period, neural crest cells, mesenchyme, and epithelium communicate with each other in a temporally dynamic manner to regulate regional identity, differentiation of progenitors into specific cell types, and morphogenesis of the future intestinal tract (Le Guen *et al.*, 2015; Roberts *et al.*, 1998). While these interactions have been the subject of scrutiny for decades, there is a growing appreciation for the molecular mechanisms involved (Chevalier *et al.*, 2021b; Huycke *et al.*, 2019; Pawolski and Schmidt, 2020; Prummel *et al.*, 2019; Prummel *et al.*, 2020; Sukegawa *et al.*, 2000; Uesaka *et al.*, 2013).

Both mouse and human intestines are functional at birth but undergo rapid growth and maturation postnatally. This includes the establishment of the intestinal stem cell niche, colonization by microbiota, and maturation of various epithelial cell types. In contrast, the intestine of zebrafish larvae intestine is not considered functional until the fifth day of embryogenesis, with additional growth and maturation that occurs during the next 4 weeks (to 33 dpf) that increases both the size and looping that is characteristic of the adult tract (Li *et al.*, 2020)

Interstitial cells of Cajal

Any of the cell types that lies within or between the smooth muscle layers of the gut could be called interstitial cells, but only one type takes the namesake of Ramon y Cajal. Ramon y Cajal was a Spanish neuroscientist and an artist in the late 19th and early 20th century. He produced detailed neuroanatomical drawings which included the neural networks of the brain and the digestive tract. Cajal was an expert with the Golgi method, a silver staining technique that enabled him to visualize cell morphology (Cajal, 1909). The Golgi method highlighted a cell population between the enteric nervous system and the bulk smooth muscles cells in the rabbit small intestine and Cajal named them ICC (Keith, 1915).

ICC are found between and within the smooth muscle layers of the digestive tract from the esophagus to the inner sphincter region of the anus in humans and mice and from the stomach to the cloaca in chick (Faussone-Pellegrini and Cortesini, 1985; Hagger *et al.*, 1998; Torihashi *et al.*, 1999a; Yun *et al.*, 2010) (Chevalier *et al.*, 2020). ICC have a small cell body and can be either bipolar or multipolar with several long processes often branching out into secondary and tertiary extensions (Hanani *et al.*, 2005). The cells sometimes form networks, and at the ultrastructural level contain numerous mitochondria (Faussone-Pellegrini, 1985; Faussone Pellegrini, 1984; Komuro, 2006), and are coupled by gap junctions to other ICC and smooth muscle cells (Ball *et al.*, 2012; Christensen, 1992; Komuro, 2006). The distribution of ICC along the digestive tract of zebrafish has not been fully described though they are reported throughout the mid intestine. Two layers of ICC were identified; one with multipolar cells located between the longitudinal and circular smooth muscle layers, and one with simple bipolar cells located deep in the circular muscle layer (Rich *et al.*, 2007).

ICC are electrically active cells that produce and propagate the electrical slow wave along the digestive tract. This electrical wave is conducted to adjacent smooth muscle cells to coordinate phasic contraction and peristalsis of the gut. (Faussone Pellegrini *et al.*, 1977; Huizinga *et al.*, 1995; Langton *et al.*, 1989; Ördög *et al.*, 1999; Rumessen *et al.*, 1982; Thuneberg, 1982; Ward *et al.*, 1994). In addition to their pacemaker function, ICC transduce inhibitory and excitatory motor neuron input from the enteric nervous system to smooth muscle cells of the gut, thereby playing a fundamental role in the process by which the nervous system regulates gut motility (Burns *et al.*, 1996; Hirst *et al.*, 2002; McErlain *et al.*, 2018; Sung *et al.*, 2018; Ward *et al.*, 2000). The physiological roles of ICC are critical to a proper functioning gut and dysfunction or loss of ICC contribute to a broad range of disorders, including diabetic and idiopathic gastroparesis (Faussone-Pellegrini *et al.*, 2012; Grover *et al.*, 2011; Ördög, 2008), intestinal pseudo-obstructions (De Giorgio *et al.*, 2004), Hirschsprung disease (Chen *et al.*, 2014), inflammatory bowel diseases (Porcher *et al.*, 2002; Rumessen *et al.*, 2011), slow transit constipation (He *et al.*, 2000; Lyford *et al.*, 2002) and others (Sanders *et al.*, 2002; Vanderwinden and Rumessen, 1999).

The origin of ICC

ICC originate from mesodermal mesenchyme, though this notion was controversial for nearly a century. ICC are difficult to identify because they share morphologic characteristics with neurons, glia, smooth muscle cells, and fibroblasts and therefore comparative studies generated conflicting conclusions. These initial studies, nicely summarized by Huizinga *et al*., were hampered by lack of a marker to definitively identify ICC (Huizinga *et al.*, 2013).

The field advanced when it was recognized that ICC express KIT which functionally contributes to ICC development, differentiation, and survival (Chabot *et al.*, 1988; Geissler *et al.*, 1988; Huizinga *et al.*, 1995). KIT is a tyrosine kinase receptor, encoded by the *KIT* gene. KIT is expressed on various cell types in addition to ICC, including mast cells, hematopoietic progenitors, melanocyte progenitors, and differentiated melanocytes (Lennartsson and

Ronnstrand, 2012). KIT is activated by its ligand, stem cell factor (SCF), also known as steel factor. SCF is expressed by enteric neurons and to a lesser extent, smooth muscle (Horváth *et al.*, 2006; Lecoin *et al.*, 1996; Torihashi *et al.*, 1996; Yamataka *et al.*, 1995). Interaction of SCF with the KIT receptor triggers production of signaling molecules that promote cellular functions including growth, migration, and differentiation (Rottapel *et al.*, 1991). SCF is produced in two forms, one secreted and one membrane bound. Each form is likely to have different actions or potency at the receptor (Lennartsson and Ronnstrand, 2012).

With the recognition that ICC express KIT, Lecoin *et al.,* (1996) addressed the controversy of ICC's origin with interspecies chimeras (Lecoin *et al.*, 1996). The major hypothesis tested through experimentation was whether KIT positive cells were of neural crest origin. They grafted quail vagal neural crest into chick embryos. In the resulting chimera, enteric innervation was of quail origin. ICC were identified by a chicken-KIT nucleic acid probe that cross-reacted with the quail *KIT* gene product. LeCoin reported that *in situ* hybridization of chimeric bowels showed that all KIT-positive cells were chick and not quail derived, and concluded they were not of neural origin (Lecoin *et al.*, 1996). Chick cells can be distinguished from quail cells using Feulgen staining or an antibody against a perinuclear antigen, QCPN (for Quail non-Chick Peri-Nuclear), though it is unclear to us which method was used to distinguish quail cells from chick cells in these experiments. The conclusion was supported by additional experiments culturing aneural chick guts on a chorioallantois membrane of quail. Typical ICC, as defined both at the EM level and by their expression of KIT receptor developed in the gut wall in the complete absence of enteric innervation (Lecoin *et al.*, 1996).

Evidence supporting a mesenchymal origin for ICC in the murine gastrointestinal (GI) tract was also presented by Young *et al.,* in the same year (Young *et al.*, 1996). Using antibodies specific for ICC or neurons they showed that ICC developed in intestinal transplants taken before the arrival of neural crest cells. Identical experiments using transplants taken later during development after arrival of neural crest cells contained both ICC and neurons.

Work from both groups is consistent with a non-neural origin of KIT positive cells early in development, leaving many to conclude that ICC originate solely from the mesodermal mesenchyme (Lecoin *et al.*, 1996; Young *et al.*, 1996). More recent work has shown a second source for gut mesenchymal tissue, the GI coelomic epithelium, which arises from an epithelial-mesenchymal transition that occurs early in development in the mouse (Carmona *et al.*, 2013). A subset of the Wt1 lineage of cells, representing coelomic origin, go onto express KIT and anoctamin 1 (ANO1), another marker of ICC (Gomez-Pinilla *et al.*, 2009). Yet another source for ICC are cells originating in the ventral part of the hindbrain that contribute to visceral organogenesis. This cell population has been named ventrally emigrating neural tube (VENT) cells (Dickinson *et al.*, 2004). VENT cells are multipotent, giving rise to neurons, glial cells, ICC, and epithelial cells in the chick stomach and duodenum (Sohal *et al.*, 2002). In summary, ICC primarily derive from mesodermal mesenchyme, but other sources such as coelomic and VENT cells are likely to contribute slightly later during development to the ICC population. Whether a differential origin creates distinct progenitor populations and/or contributes to morphological or functional differences amongst ICC has not been reported.

Differentiation of ICC - the early embryonic period

In the developing digestive tract, KIT positive cells first emerge outside of the differentiating circular muscular layer (beginning at d7 in chick; E12 in mice and week 7-9 in humans (Faussone-Pellegrini *et al.*, 2007; Iino *et al.*, 2020; Kenny *et al.*, 1999; Keshet *et al.*, 1991; Klüppel *et al.*, 1998; Lecoin *et al.*, 1996; Radenkovic, 2012; Radenkovic *et al.*, 2010b; Torihashi *et al.*, 1997; Wallace and Burns, 2005; Wester *et al.*, 1999; Wu *et al.*, 2000). KIT positive cells emerging during the early embryonic period are more abundant, more widely distributed, and morphologically different from mature ICC (Klüppel *et al.*, 1998; Radenkovic, 2012, Radenkovic *et al.*, 2010a; Radenkovic *et al.*, 2010b; Roberts *et al.*, 2010; Torihashi *et al.*, 1997). The putative ICC progenitor cells appear with a small cell body, large nucleus, and numerous but short cellular processes (Radenkovic *et al.*, 2018). Later experiments revealed that these cells, in addition to expressing KIT, may also express Ano1, PDGFα and PDGFβ (Chevalier *et al.*, 2020; Huang *et al.*, 2009; Kurahashi *et al.*, 2008). Ano1 is a calcium-activated chloride channel considered to be critical for mature ICC function (Gomez-Pinilla *et al.*, 2009) (Huang *et al.*, 2009), while PDGFα and PDGFβ are growth factor receptors, implicated in fibroblast and smooth muscle development (Chen *et al.*, 2013) and neural crest migration (Shellard and Mayor, 2016).

Distribution of KIT positive progenitors is broader when compared with distribution of mature ICC (Klüppel *et al.*, 1998; Radenkovic, 2012, Radenkovic *et al.*, 2010a; Roberts *et al.*, 2010). Whether their distribution arises through de novo differentiation of mesenchymal cells or expansion of preexisting progenitor population is not known. Single-cell analysis of digestive organs during embryogenesis identified multiple conserved and transcriptionally distinct mesenchymal cell populations which support the possibility of an ICC specified mesenchymal cell population (Loe *et al.*, 2021). Single cell RNA analysis focusing on embryonic ICC progenitors, such as have been done in adults (Wright *et al.*, 2021), has the potential to bring a better understanding of the cells that give rise to KIT positive progenitors and the signals influencing their emergence.

Given that embryonic progenitor cells express KIT, it is a natural question whether ICC maintenance, differentiation, or both rely on KIT signaling. Mice with defects in KIT signaling have normal ICC networks at birth, which suggests that KIT signaling is not required for lineage determination of ICC during early embryogenesis (Klüppel *et al.*, 1998; Thuneberg, 1990). This was shown using W banded (W*bd*) mice which have a genomic rearrangement of chromosome 5 resulting in inversion of *KIT* and a loss of KIT expression during embryogenesis (Thuneberg, 1990). At postnatal day 5 KIT expression was absent but apparently normal ICC distributions were observed using methylene blue staining (Thuneberg, 1990). Methylene blue staining is not specific to ICC. In a different set of experiments, Bernex and coworkers inserted a lacZ gene into the first exon of *KIT*, creating a null allele, W*lacZ*. LacZ transgene expression overlapped Kit expression in heterozygous $W^{lacZ/+}$ embryos in the colon of E16.5 animals (Bernex *et al.*, 1996). The pattern of LacZ expression LacZ was the same in $W^{acZ/+}$ and $W^{lacZ/lacZ}$ embryos (Bernex *et al.*, 1996), but ICC distributions were not assessed independently from LacZ. Now that additional markers of ICC are available, it would be interesting to revisit the question of whether KIT signaling is

required during early embryogenesis following ICC progenitors by Ano1 immunoreactivity (Chevalier *et al.*, 2020; Huang *et al.*, 2009; Kurahashi *et al.*, 2008).

Differentiation of ICC – late embryonic period after 15 days

During the late embryonic period, the number of KIT positive progenitor cells declines, and some cells begin to express smooth muscle markers. For example, in the colon some cells are immunoreactive for γ-enteric actin (Torihashi *et al.*, 1999a), an actin isoform associated with smooth muscle, and cells in the small intestine are immunoreactive for vimentin or desmin, markers of immature smooth muscle (Bornemann and Schmalbruch, 1993; Torihashi *et al.*, 1997; Ward and Sanders, 2001). The longitudinal layer of smooth muscle appears (Klüppel *et al.*, 1998; Radenkovic, 2012; Torihashi *et al.*, 1997) by d13 in the chick (Graham *et al.*, 2017; Shyer *et al.*, 2013), E16.5 in the mouse (Chevalier *et al.*, 2021a), week 11 in humans (Wallace and Burns, 2005), and 80-98 hpf zebrafish (Olden *et al.*, 2008; Seiler *et al.*, 2010). Many of the remaining KIT positive cells in the myenteric plexus region and in the muscular layers take on a distinct morphology and distribution characteristic of mature ICC. (Abramovic *et al.*, 2014; Iino *et al.*, 2020; Radenkovic, 2012, Radenkovic *et al.*, 2010a; Radenkovic *et al.*, 2010b; Torihashi *et al.*, 1997; Ward *et al.*, 1997). They extend multiple fine processes and form mature networks indistinguishable in appearance from those in adults, and the electrical slow wave becomes detectable (Roberts *et al.*, 2010; Torihashi *et al.*, 1997; Ward *et al.*, 1997). The slow wave is a repeating depolarizing and repolarizing oscillation of membrane potential. It's not an action potential and it functional organizes phasic contractions in GI muscles (Sanders, 2019) The slow wave is initiated in ICC and is transmitted to smooth muscle, where it is typically measured. In mice the slow wave emerges in the stomach and proximal small intestine before birth (by E19), soon after birth in the ileum, and after several days in the colon and subsequently the amplitude and frequency increases (Ward *et al.*, 1997).

Although ICC lineage determination during early embryogenesis is KIT independent (Klüppel *et al.*, 1998; Thuneberg, 1990; Bernex *et al.*, 1996)), during late embryogenesis, KIT signaling is crucial for normal ICC development. At postnatal day 15, W^{bd}/W^{bd} mice showed a marked reduction in methylene blue positive cell density, indicating that KIT function is necessary for expansion or specification of ICC during this time period(Klüppel *et al.*, 1998). Additionally, treatment with neutralizing kit antibodies, in organotypic culture from murine gut and in newborn animals, has reduced ICC number, disrupted ICC networks and slow wave, rendered muscles electrically quiescent, and altered gut motility and contractility (Torihashi *et al.*, 1995; Sato *et al.*, 1996; Ward *et al.*, 1997; Torihashi *et al.*, 1999b; Maeda *et al.*, 1992; Beckett *et al.*, 2007).

ICC heterogeneity

Mature ICC are a heterogenous population with subclasses based upon distribution, cell morphology, connectivity, and function (Hanani *et al.*, 2005; Huizinga *et al.*, 2011; Koh *et al.*, 1998; Parsons and Huizinga, 2020; Sanders *et al.*, 2006; Thomsen *et al.*, 1998; Yang *et al.*, 2012). The molecular factors influencing development or identification of ICC subtypes are not established.

The most prominent and best studied ICC are located between the circular and longitudinal muscles layers, referred to as myenteric ICC (ICC-MY), that form a network surrounding the neuronal myenteric plexus (Komuro, 2006; Sanders *et al.*, 1999). ICC-MY are observed in every organ of the GI tract, from the esophagus to the colon. A second ICC network is observed in the deep muscular plexus region of the small intestine, located in the innermost layer of circular smooth muscle (ICC-DMP) (Sanders *et al.*, 1999). Single ICC are distributed throughout the circular smooth muscle layer and therefore are termed intramuscular (ICC-IM). Finally, a more dense population of un-connected ICC are observed close to the submucosal border of the colon (ICC-SM) (Gomez-Pinilla *et al.*, 2009; Komuro, 2006; Sanders *et al.*, 1999). More detailed descriptions of ICC classification are available (Farrugia, 2008; Vanderwinden and Rumessen, 1999; Ward and Sanders, 2001). Subtypes of ICC are differentially dependent on KIT signaling. ICC-MY and ICC-IM develop before birth in the mouse and require KIT signaling (Burns *et al.*, 1996; Torihashi *et al.*, 1997), whereas ICC-DMP and ICC-SM develop postnatally and are less reliant on KIT signaling (Faussone Pellegrini, 1984; Torihashi *et al.*, 1995; Ward *et al.*, 1997). For example, in Sl/Sl^{d} or W/W^{v} mice, which have reduced KIT signaling, ICC-DMP of the small intestine are not affected (Iino *et al.*, 2020; Kwon *et al.*, 2009). Similarly, ICC-SM in the subserosal layer of the colon are visible in W/W^{v} mice (Tamada and Kiyama, 2015).

Interestingly, ICC-IM and ICC-MY in the large intestine, small intestine, stomach, and cecum require expression of ETV1. ETV1 is an ets family transcription factor and a master regulator of ICC. ETV1 acts as part of an ERK-ETV1-KIT positive feedback loop to stimulate *KIT* transcription via enhancer binding (Hayashi *et al.*, 2015; Ran *et al.*, 2015; Tamada and Kiyama, 2015). $Etv1^{-/-}$ mice show a significant loss of KIT-positive ICC-IMs and ICC-MYs. In contrast, ICC-DMPs and ICC-SM in the small and large intestine respectively are preserved, consistent with the kit independence and absence of ETV1 expression in these ICC subtypes (Tamada and Kiyama, 2015).

Much less studied is the fact that some ICC populations may have different or additional requirements during development. Kondo *et al.,* examined development of later developing ICC and showed expression of leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1) in ICC-DMP and ICC-SM (Kondo *et al.*, 2015). LRIG1 knock out mice lack ICC-DMP and ICC-SM and have slower transit in the small intestine.

ICC-IM may also require expression of natriuretic peptide receptor B (NPR-B). $Npr2^{slw/slw}$mice are a spontaneous mutant mouse strain, known also as a short-limbed dwarfism (*SLW*) mouse. Mice homozygous for SLW ($Npr2^{slw/slw}$) are defective in NPR-B function due to a frameshift mutation in *Npr2*, particularly in the exon-8 encoding the region present just under the transmembrane domain (Sogawa *et al.*, 2010). The intestines of preweaning $Npr2^{slw/slw}$ mice showed a clear reduction in the number of ICC-IM (Sogawa-Fujiwara *et al.*, 2020).

Transcription profiling provides the opportunity to identify novel genes expressed in ICC that will contribute to understanding ICC development and function as well as molecular markers for ICC subtypes. Transcriptome profiling during development may identify molecular programs and switches determining ICC progenitor fate. An ICC transcriptome from GFP labeled mouse intestine identified novel markers, growth factors, transcription factors,

ion channels, and ion transporters (Lee *et al.*, 2017). Unique ICC markers thrombospondin-4 and hyperpolarization activated cyclic nucleotide gated K+ channel (*Thbs4* and *Hcn4*) were identified, as was expression of ten transcriptional variants of *Ano1* (Lee *et al.*, 2017). The role(s) for *Thbs4* or *Hcn4* in ICC physiology, relating to motility, development, or turnover, would be interesting but has not been reported. Interestingly, two zebrafish *hcn4* genes have been characterized and pharmacologic inhibition in developing embryos slows heart rate (Liu *et al.*, 2022). The effects of *Hcn4* inhibition on motility patterns in 7 dpf embryos would indicate a functional role in ICC. A more recent publication examined gene expression in 5572 smooth muscle cells, 372 ICC cells, and 4805 platelet derived growth factor alpha cells isolated from colonic tissue surgery in 15 patients (Schneider *et al.*, 2023). Platelet derived growth factor alpha cells are a second type of intestinal interstitial cell that works with smooth muscle and interstitial cells of Cajal to coordinate motility patterns. Cell type was identified based upon expression of specific genes such as *KIT* and *ANO1* for ICC. These authors noted that expression of the mechano-sensitive ion channel *PIEZO2* in ICC but not the other cell types. *piezo 2b* is the zebrafish homolog for human *PIEZO2* and a functional role for *piezo 2b* was shown in the touch response for zebrafish embryos (Faucherre *et al.*, 2013). If intestinal motility patterns in 7 dpf zebrafish are altered after *piezo 2b* knockdown, a functional role in ICC would be indicated. Apart from ion channels, a common transcriptional regulatory pathway for smooth muscle, ICC, and platelet derived growth factor alpha cells, three cell types involved in pacing and regulating smooth muscle contraction, was identified (Wright *et al.*, 2021). A better understanding of this transcriptional regulatory pathway during development or cell turnover in mature tissue may help to identify the mechanisms determining progenitor cell development, expansion, and differentiation to mature ICC subtypes. Manipulating or reprograming regulatory pathways will facilitate long-term rehabilitation of dysmotility.

To summarize, the heterogeneity of ICC is evident through development. ICC-MY and ICC-IM develop before birth in mice and have a strong requirement for KIT-signaling. ICC-DMP and ICC-SM develop postnatally and are less dependent on KIT signaling. ICC-MY and ICC-IM require ETV1 function, and ICC-IM require NPR-B. ICC-DMP and ICC-SM require LRIG1. It is important to understand the relationships of ICC subtypes and their contributions to overall gut function. ICC heterogeneity is understudied in zebrafish. The zebrafish digestive tract is simpler along its length, the muscularis mucosae is absent, and it is less complex (Wallace *et al.*, 2005; Wallace and Pack, 2003). Zebrafish are likely to have fewer ICC subtypes and therefore may be a good system to determine the functional contributions of distinct ICC populations on GI motility. A thorough morphological and molecular characterization of ICC subtypes in zebrafish remains to be done.

Enteric nervous system influences on ICC development

Development of the enteric nervous system precedes ICC development in the mouse intestine (Wu *et al.*, 2000). Since enteric neurons express the kit ligand SCF, and ICC-MY and ICC-IM are juxtaposed to enteric neurons, it is expected that ICC development would depend upon enteric neurons. However, there are conflicting reports. Knockout mice lacking glial cell line-derived neurotrophic factor signaling (*GDNF-/-* mice) do not develop enteric neurons and express normal ICC populations (Uesaka *et al.*, 2013; Ward *et al.*, 1999). Different mouse lines lacking enteric neurons, ls/ls and r*et-/-*, have disrupted development of ICC-MY, either distally, or along the entire length of the intestine (Wu *et al.*, 2000). Other ICC populations do not appear to depend on SCF produced by enteric neurons (Uesaka *et al.*, 2013; Wu *et al.*, 2000).

Reconciling this data is difficult. One potentially important difference between these mutant mice is the presence and or localization of enteric neural progenitors. They are lost in the r*et-/-* mice, retained in *GDNF-/-* mice, and have altered localization in ls/ls mice (Uesaka *et al.*, 2013; Wu *et al.*, 2000). While enteric neurons are not required for ICC development, neural progenitors may support ICC-MY development. Three ENS progenitor populations have been identified in zebrafish and examining ICC development when ENS progenitors are lacking would contribute to understanding the relationships between ICC and ENS during development (Taylor *et al.*, 2016).

Smooth muscle influence on ICC development

Smooth muscle development is a major regulator for patterning of the digestive tract with molecular and mechanical forces driving morphogenesis, resulting in orthogonal circular and longitudinal smooth muscles cells (Huycke *et al.*, 2019). As the circular smooth layer differentiates there is a notable transition in the gut; the associated extracellular matrix drives the existing enteric nervous system network towards a highly oriented morphology (Chevalier *et al.*, 2021b). Whether these same factors influence ICC development is an unanswered, but intriguing question.

Maintenance of ICC

The ICC distribution and networks that develop late in the embryonic period remain largely unchanged into adulthood. ICC are maintained through a balance of survival/trophic/growth factors, cell loss, and cell replacement by adult ICC progenitors (Bardsley *et al.*, 2010; Hayashi *et al.*, 2013; Horváth *et al.*, 2006; Horváth *et al.*, 2005; Ning *et al.*, 2010). Adult ICC progenitors have been isolated from intestinal tissues of adult mice and characterized as $KIT^{low}CD44^{+}CD34^{+}Insr^{+}Igf1r^{+}$ cells (Lorincz *et al.*, 2008). These cells are capable of self-renewal in organotypic cultures (Bardsley *et al.*, 2010; Lorincz *et al.*, 2008). The proliferative state of ICC progenitors can be stimulated by IGF-1 or soluble SCF (Bardsley *et al.*, 2010; Lorincz *et al.*, 2008). IGF-1 may act directly on the progenitors and/or regulate production of SCF from smooth muscle and enteric neurons (Bardsley *et al.*, 2010; Horváth *et al.*, 2006; Lorincz *et al.*, 2008; Yang *et al.*, 2017; Zhang *et al.*, 2014). IGF-1 may also induce SCF production from ICC progenitors in an autocrine loop because KIT neutralizing antibodies partially inhibited IGF-I-induced proliferation (Bardsley *et al.*, 2010). Proliferation of ICC progenitors is also regulated by 5-HT(2B) signaling as $Htr2b^{-/-}$ mice show reduced proliferation of ICC-MY (Tharayil *et al.*, 2010; Wouters *et al.*, 2007).

Interestingly, it does not appear KIT signaling is required to maintain the basal proliferation of ICC progenitors. Bardsley and co-workers examined this possibility because human patients with GIST relapse after treatment with tyrosine kinase inhibitors (Bardsley *et al.*, 2010). Using a mouse with an activating mutation in KIT, they isolated putative KIT progenitor cells from adult

tissues then incubated them in tyrosine kinase inhibitors. These cells, termed KIT^{low}, expressed just 10% of normal KIT on the cell surface, yet were able to develop into mature ICC (Bardsley *et al.*, 2010). ICC progenitors also differentiate into mature ICC; first into KIT^{+}CD44^{+}CD34^{+}Insr+Igf1r+ intermediate cells with ICC morphology, and then into mature slow wave producing, network forming KIT^{+}CD44^{+}CD34^{-}Insr^{-}Igf1r^{-} ICC (Lorincz *et al.*, 2008). Membrane-bound SCF may drive this differentiation because adult *Sl/Sl*^{d} mice, which express only soluble SCF, show decreased numbers of intermediate and mature ICC in adulthood (Bardsley *et al.*, 2010). Stimulation of ICC differentiation from the progenitor to mature phenotype by membrane bound SCF has not been demonstrated directly.

The potential that membrane bound SCF drives differentiation of adult ICC progenitors implies a role for KIT signaling in the ongoing maintenance of ICC networks. It is unclear whether adult progenitors support the differentiation of all the different ICC subtypes seen in vivo. In adulthood, ICC progenitor proliferation can be supported by, but is not dependent upon, KIT signaling. In contrast, ICC differentiation and thus maintenance of the networks is likely to partly depend on KIT signaling. Transcriptomics is beginning to provide a molecular description of ICC in adult tissues but the transcriptome for ICC subtypes in adults, the molecular programs during development, and the molecular signaling driving differentiation of adult ICC progenitors is not yet clear (Foong *et al.*, 2022; Lee *et al.*, 2017).

Future directions

Examining ICC progenitors in intact organisms, with all of the associated complexity, would be beneficial. The zebrafish model may fill this niche. Zebrafish develop externally and embryogenesis can be directly observed in living animals. During later development and organogenesis, larvae are relatively transparent, and pigmentation mutants *casper* and *albino* extend the timespan when the GI tract can be easily visualized. ICC have been identified in the zebrafish GI tract with antibodies to KIT and ANO1 (Ball *et al.*, 2012; Rich *et al.*, 2007; Uyttebroek *et al.*, 2013). Genome duplication in the teleost lineage has generated two paralogs of the KIT receptor (*kita* and *kitb)* and ANO1 (*ano1a* and *ano1b*). All 4 genes are expressed in the zebrafish GI tract (Nikaido *et al.*, 2023; Rich *et al.*, 2007). Retention of duplicate gene copies often leads to functional divergence. It is possible that zebrafish orthologues differentially identify ICC progenitors and/or differentiated subtypes of ICC. Generation of gene reporter fish that allow direct observation of ICC lineage progression is feasible in intact animals. A better understanding of ICC origin and maintenance for all ICC subtypes will contribute to understanding GI motility.

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