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# Immunohistochemical characterization of interstitial cells of Cajal (ICC) in the mouse intestine using whole mount preparations – with particular reference to their density, morphological features, and network patterns

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**Summary. The aim of this study was to evaluate the distribution pattern of the different subtypes of the interstitial cells of Cajal (ICC), to understand their roles in gastrointestinal motility and interaction with the enteric nervous system depending on their location. Based on morphological analyses using the immunohistochemistry of whole mount preparations of mouse intestine, we investigated the normal local density and morphological features of ICC and statistically compared the number of the cells between the mesenteric and anti-mesenteric sides, and between the proximal and distal sites. Density gradients were shown in ICC associated with the deep muscular plexus, the myenteric plexus in the large intestine, and the longitudinal muscle. However, such changes in cell density were not always proportional to other subtypes, even in the same part of the intestine. As the pacemaker cells for intestinal motility, dense and seamless network connections were shown in ICC associated with the myenteric plexus in the small intestine and ICC associated with the submuscular plexus in the large intestine without any gradient of cell density, in contrast with the difference of morphological network patterns between proximal and distal sites. Thus, we conclude that the distribution patterns of ICC differ in cell density, morphological features, and network patterns, depending on the individual subtype and site of the intestine. Therefore, careful studies on the quantification and distribution of ICC under normal conditions should be important in diagnosing clinical disorders related to abnormal distribution of ICC.**

**Keywords: Interstitial cells of Cajal (ICC), subtype, distribution, intestines, mouse**

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## Introduction

Transport and absorption of food and fluid are highly regulated by constitutive gastrointestinal motility. It has been

shown that several components of the regulatory mechanism of smooth muscle movement may be involved (Sanders *et al.* 2012).

As the main control system for gastrointestinal motility, the enteric nervous system (ENS) has been studied in great detail (Costa *et al.* 1971; Furness and Costa 1971, 1980). Quantitative studies of ENS have been based on morphological observations of neurons in the myenteric plexus (Irwin 1931; Gabella 1971). These studies have revealed that the distribution of neurons in the gut is not uniform but differs in different parts. For instance, the large intestine contains a greater density of neurons than the small intestine and their mesenteric sides have a greater density than the anti-mesenteric sides. Therefore, gastrointestinal motility differs in different parts of the alimentary tract, mainly due to the uneven distribution of the ENS. For example, in the small intestine, peristaltic movement is uni-directional (Cannon 1902) and segmentation motor activity is a movement that facilitates absorption of nutrients. In human, the movement of the large intestine has been classified as non-propulsive segmentation, propulsive, and retropulsive (antiperistalsis) movements (Ritchie 1970). In mammals, antiperistalsis is limited to the proximal part of the colon (Elliott and Barclay-Smith 1904).

Another control system, the interstitial cells of Cajal (ICC), have been shown to play an important role in gastrointestinal motility, by many studies (Huizinga *et al.* 1995; Sanders *et al.* 2014). Morphological studies using electron microscopy have elucidated the microstructure of ICC (Imaizumi and Hama 1969; Komuro 1989, 1999, 2006; Komuro *et al.* 1999; Yamamoto 1977), and the discovery of their specific marker, the tyrosine kinase receptor (c-Kit), has also contributed to the distinguishing of ICC from other interstitial cells (Maeda *et al.* 1992). ICC are found in the smooth muscle layer of the gut and classified into several subtypes, according to their localization in the muscle layers (Fig. 1). The subtypes are ICC associated with deep muscular plexus of the small intestine (ICC-DMP), myenteric (Auerbach) plexus (ICC-MP), and submuscular plexus of the colon (ICC-SMP), and ICC located within the circular muscle layer (ICC-CM) and within the longitudinal muscle layer (ICC-LM) (Thuneberg 1982, 1989). Physiological investigation of ICC have also shown dramatic progress due to the pacemaker hypothesis proposed by Thuneberg (Thuneberg 1982, 1989). The major functions of ICC are as pacemakers of gut motility and as intermediates in the transmission of messages between ENS and smooth muscles. Moreover, the function of each subtype of ICC does not seem to be the same depending on the sites where they are localized. It has also become clear that ICC and the ENS almost always work together (Huizinga *et al.* 2011).

However, there have not been any systematic studies on the distribution of ICC subtypes throughout the intestine. Further, many intestinal disorders have been reported to be related to the number or density of ICC. Basic information about ICC, including their actual function in these diseases is not sufficient to be used as diagnostic criteria in each intestinal dysfunction. Therefore, it is very important as basic information, to understand the normal distribution and the features of the normal morphological network in detail, for each subtype of ICC, in different locations of the intestine, in the context of their function in gastrointestinal motility and interaction with ENS.

In the present study, as a systematic study on the distribution of ICC subtypes throughout the intestine, we evaluated the overall distribution pattern of each ICC subtype in the mouse intestine. Based on morphological analyses using specific antibodies to identify ICC (Komuro 1999; Ward *et al.* 1994) and ENS (Fu *et al.* 2013; Iino and Horiguchi 2006), we measured the local density of ICC and compared the difference in the number of ICC between mesenteric and anti-mesenteric sides, and between proximal and distal sites, in the mouse intestine. We also discuss whether the quantitative features of each subtype of ICC may correspond to their possible physiological roles in the mouse intestine.

## Materials and Methods

### *Animals and Ethics*

C57BL/6J female mice (6–8 weeks old) were purchased from CLEA Japan (Tokyo, Japan) and used for all experiments designed in this study, which were approved by the Ethical Review Committee of Animal Experiments at Tokyo Women's Medical University (TWMU), approval number is AE18–71 (Mar 31, 2018). Mice were anesthetized using an intraperitoneal injection of ketamine (87 μg/g body weight),

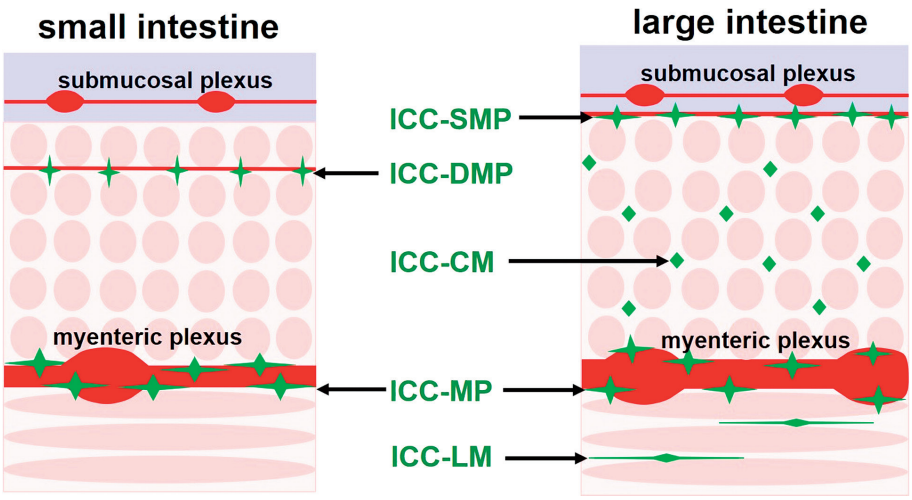


Fig. 1. Subtypes of ICC and their Location. A diagram showing the cross-section of small and large intestine (from submucosal layer to muscle layer) and various subtypes of ICC (in green) and the enteric nervous system (in red). ICC-SMP are present on the inner aspect of the circular muscle layer in the large intestine. ICC-DMP represent a population of cells within the specialized deep muscular plexus region of the small intestine. ICC-CM are located within the circular muscle in the large intestine. ICC-MP are associated with the myenteric (Auerbach) plexus and located between the circular and longitudinal muscular layers throughout the gastrointestinal tract. ICC-LM are located within the longitudinal muscle in the large intestine. ICC-DMP: ICC associated with deep muscular plexus, ICC-SMP: ICC associated with submuscular plexus, ICC-CM: ICC located within the circular muscle layer, ICC-MP: ICC associated with myenteric (Auerbach) plexus, ICC-LM: ICC within the longitudinal muscle layer

xylazine (13 μg/g body weight), and metacam (1 μg/g body weight) and were sacrificed by cervical dislocation. All handling and care of animals was carried out in accordance with the guidelines of the Institute of Laboratory Animals for Animal Experimentation and approved by the Animal Care and Use Committee of TWMU.

### *Tissue preparation*

Tissue samples, including the small intestine, colon, and rectum were removed from at least three mice ($n \geqq 3$), placed into phosphate buffered saline (PBS; pH 7.2). The length and the diameter of each intestine was measured and the fecal matter was washed out promptly. For inhibition of contractile activity in the intestines, the intestines were placed into saline solution containing papaverine (0.2 mg/mL), a calcium antagonist, for 10 min at 37°C and then rinsed with PBS. The intestines were inflated with acetone to the original size and fixed in ice-cold acetone for 20 min. After rinsing in PBS, the intestines were opened along the mesenteric border, the posterior side in rectum, and pinned to a dissecting dish. Lengths of 3 cm of the selected regions (jejunum: the proximal side, ileum: the distal end, colon: proximal colon (1 cm distal from the ileocecal valve), and rectum: the distal end) were prepared and oriented as shown in Fig. 2. In this study, we called the preparations of the jejunum and ileum as the proximal and distal sites of the small intestine, respectively, and the preparations of the colon and rectum as the proximal and distal sites of the large intestine, respectively. The whole-mount preparations of the muscle layers were prepared carefully by peeling off the mucosa and submucosa with fine forceps, under a dissecting microscope.

### *Immunohistochemistry*

Whole-mount samples were placed in PBS containing 0.5% Triton X-100 for 10 min at room temperature (approximately 20 ± 5°C). Non-specific binding was blocked by incubation in 5% Block Ace (DS Pharma Biomedical, Osaka, Japan) in distilled water, for 20 min. The samples were then incubated with a rat monoclonal anti c-Kit antibody (ACK2, OriGene,

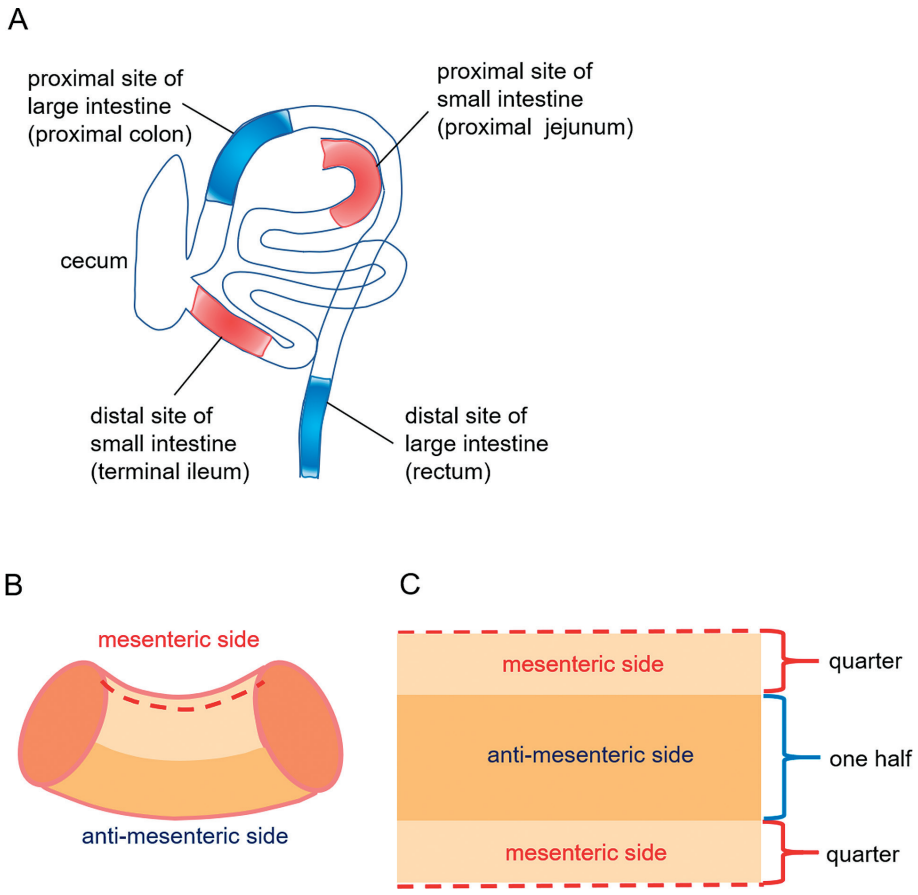


Fig. 2. Diagrams of Tissue Preparations. Constant lengths (3 cm) from proximal jejunum, terminal ileum, proximal colon, and rectum were obtained for each preparation and opened along the mesenteric border. (A, B). The whole mount preparations of the muscle layers were made after peeling away the mucosa and submucosa, which were placed in the same direction. The lateral quarter areas were regarded as the mesenteric side and the middle area was regarded as the anti-mesenteric side (C). Photomicrographs were taken in eight random fields from both the mesenteric and anti-mesenteric side of each level of ICC subtype (ICC-DMP, ICC-MP, ICC-SMP, ICC-CM, and ICC-LM) from each part of the intestine (jejunum, ileum, colon, and rectum). The horizontal direction of photomicrographs was in the direction of the long axis.

MD, USA) at a dilution of 1:100, and a polyclonal rabbit UCHL1/PGP9.5 antibody (Proteintech, IL, USA) at a dilution of 1:500, for 2 nights at 4°C, to identify ICC and ENS, respectively. After washing in PBS, the samples were further incubated overnight at 4°C with either Alexa Fluor® 488 conjugated goat anti-rat IgG at a dilution of 1:200 (Eugene, OR, USA), Cy3 conjugated goat anti-rabbit IgG at a dilution of 1:200 (Jackson Immunoresearch, PA, USA), or Hoechst 33342 at a dilution of 1:2,000 (Dojindo, Kumamoto, Japan) for nuclear staining. All antibodies were diluted in 1% bovine serum albumin in PBS. After washing with PBS, the tissues were mounted on glass slides and examined with a Zeiss LSM 510 confocal microscope (Zeiss, Germany), at 488 nm (for Alexa Fluor 488), 561 nm (for Cy3), and 405 nm (for Hoechst 33342).

### *Morphological measurement and statistical analyses*

Photomicrographs were taken for eight random fields (400× magnification) from both the mesenteric side and the anti-mesenteric side, at each level of ICC subtype localization (ICC associated with deep muscular plexus (ICC-DMP), ICC

associated with myenteric plexus (ICC-MP), ICC associated with submuscular plexus (ICC-SMP), ICC located within the circular muscle layer (ICC-CM), and ICC located within the longitudinal muscle layer (ICC-LM)), at various sites of the intestine (jejunum, ileum, colon, and rectum), with confocal microscopy. Confocal micrographs were obtained, with digital composites of Z-series, through a depth of 6–12 μm for ICC-DMP, 8–20 μm for ICC-MP, 4–16 μm for ICC-SMP, 4–30 μm for ICC-CM, and 1–5 μm for ICC-LM. Final images were constructed using the Zeiss software (Zen). The number of c-Kit/Hoechst 33342 double labeled cells were counted by examining the confocal micrographs. c-Kit positive areas were measured using ImageJ.

Data were expressed as means (standard deviation: SD) per square millimeter. As an exception, those of ICC-CM and ICC-LM were expressed as means per cubic millimeter because of the thickness of circular and longitudinal muscles in ICC-CM and ICC-LM, respectively, depending on their location. Differences in the data were evaluated by Student's *t*-test. Difference probabilities; $P < 0.05$ was regarded as statistically significant, and $P < 0.01$ was highly significant.

## Results

### *I. Typical morphological features of various subtypes of ICC*

The typical features of ICC subtype, as identified by their c-Kit immunoreactivity, at each local site are shown in Fig. 3. In the small intestine, ICC were mainly identified in two regions; the deep muscular plexus (ICC-DMP) and the myenteric plexus (ICC-MP). c-Kit immunoreactivity was rarely found in the thick sublayer of the circular muscle and in the longitudinal muscle layer. ICC-DMP were located adjacent to the nerve bundles and projected long slender processes along the deep muscular plexus (Fig. 3A). In contrast, ICC-MP formed a more complex cellular network in the meshwork of the nerve plexus (Fig. 3B). In the large intestine, four subtypes of ICC were recognized. ICC-SMP were clearly recognized as ICC associated with the submuscular plexus, but in a way, demarcating the submucosal border of the circular muscle layer. Their cell bodies were randomly distributed but connected with each other with their long slender processes (Fig. 3C). In the circular muscle layer, ICC-CM were located within the muscle bundles (Fig. 3D). They seemed to be closely associated with the nerves within the circular muscle layer that were oriented in parallel with the circular muscle bundles. Their shape was not simply bipolar, but multipolar. However, ICC-MP formed a more complex cellular network (Fig. 3E). Their cell bodies seemed to associate closely to the myenteric plexus and connected with each other with their long slender processes. Within the longitudinal muscle layer, a few ICC-LM were detected as spindle shaped or bipolar cells (Fig. 3F). They ran along the length of the muscle bundles with fewer processes but there were few nerve fiber around.

### *II. Distribution density of various subtypes of ICC*

To investigate the general distribution of ICC in the intestine and to compare the difference in the number of ICC at each site between mesenteric and anti-mesenteric sides, and between proximal and distal sites, we first counted the actual number of ICC at the determined sites in the mouse intestine, as described in Fig. 2.

#### *1) Comparison of distribution density between the mesenteric side and the anti-mesenteric side*

In general, no distinctly significant difference in the cell density of most subtypes of ICC, between the mesenteric and the anti-mesenteric side was seen. However, the cell densities of ICC-DMP in the jejunum, ICC-CM in the rectum, and ICC-MP in the rectum were significantly higher at the mesenteric side than the anti-mesenteric side (Table 1).

#### *2) Comparison of distribution density between proximal and distal sites*

There was no difference in the cell density of ICC-MP within the small intestine between the proximal and distal sites and that of ICC-SMP and ICC-CM within the large intestine, between the proximal and distal sites. In contrast, ICC-MP in the large intestine showed a significantly higher cell density at the proximal site than in the distal site, and cell density of ICC-DMP in the small intestine and ICC-LM in the large intestine was significantly higher in the distal sites than in the proximal sites (Fig. 4).

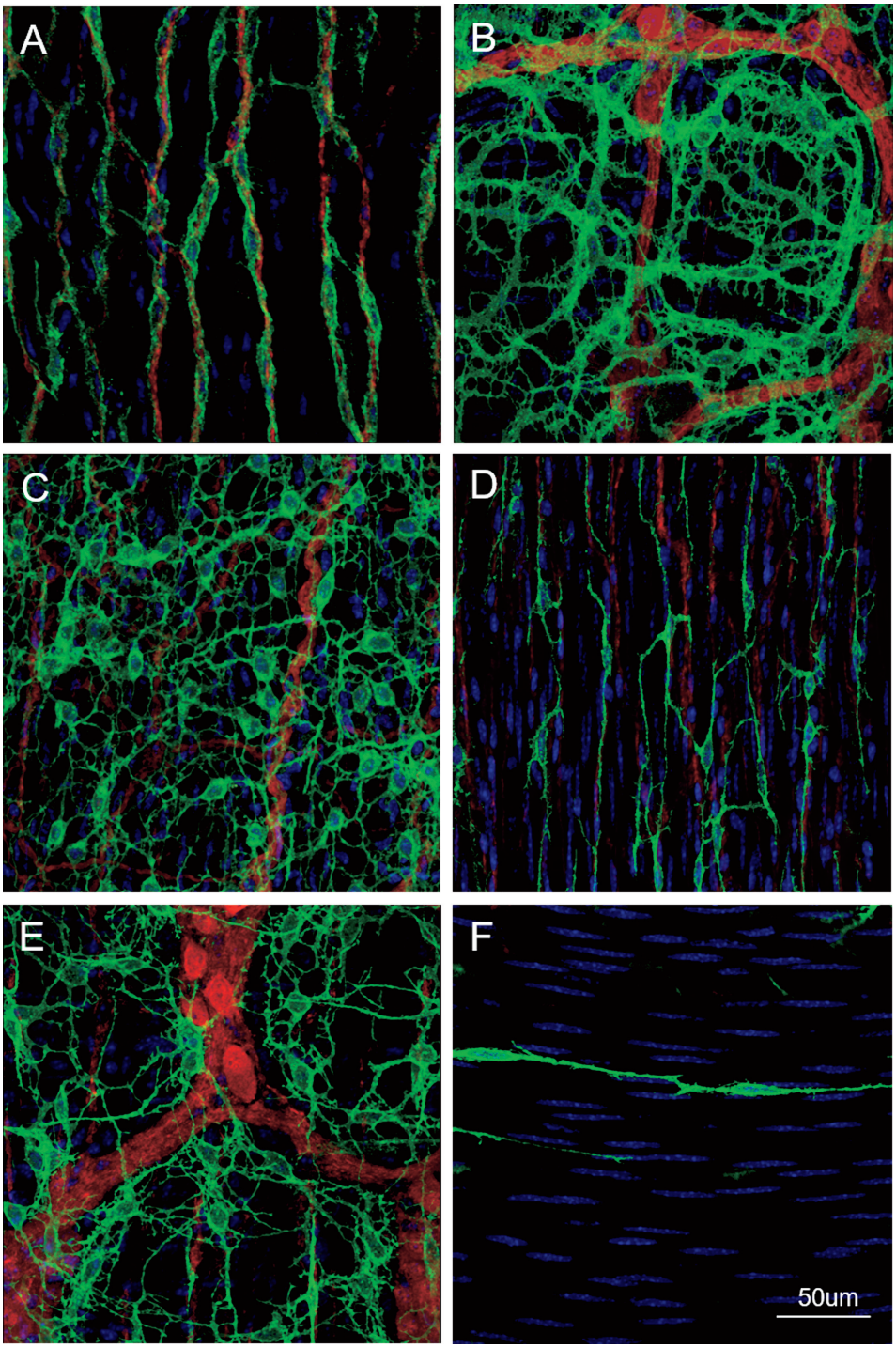


Fig. 3. Typical Features of ICC Subtypes in the Mouse Intestine. Whole-mount preparations showing c-Kit positive ICC (green) and PGP9.5 positive nerves (red). Typical features of each ICC subtype are presented as representative subtypes. In the small intestine, ICC-DMP in the ileum (A) and ICC-MP in the jejunum (B), were identified. In contrast, four subtypes of ICC were detected in the large intestine. ICC-SMP in the colon (C) and ICC-CM, ICC-MP, and ICC-LM in the rectum (D-F, respectively). Bar indicates 50 μm.

Table 1. Mean cell density of ICC in each part of the intestine and the difference between mesenteric and anti-mesenteric sides in the mouse intestine

| ICC subtype | part | mesenteric side | anti-mesenteric side | *p value* |
|---|---|---|---|---|
| **Small intestine** | | | | |
| 1. ICC-DMP | jejunum | 439 (80.4) | 388 (87.5) | 0.04 * |
| (cell/mm$^2$) | ileum | 483 (118.3) | 476 (116.3) | 0.84 |
| 2. ICC-MP | jejunum | 721 (129.8) | 686 (122.9) | 0.34 |
| (cell/mm$^2$) | ileum | 686 (130.3) | 664 (124.2) | 0.55 |
| **Large intestine** | | | | |
| 1. ICC-SMP | colon | 729 (146.5) | 713 (221.4) | 0.76 |
| (cell/mm$^2$) | rectum | 719 (116.8) | 680 (102.5) | 0.22 |
| 2. ICC-CM | colon | 36411 (15028.3) | 29965 (12808.1) | 0.11 |
| (cell/mm$^3$) †1 | rectum | 38265 (15378.5) | 29302 (14751.3) | 0.04 * |
| 3. ICC-MP | colon | 1298 (125.1) | 1247 (153.7) | 0.20 |
| (cell/mm$^2$) | rectum | 960 (169.9) | 792 (112.8) | 0.0002 ** |
| 4. ICC-LM | colon | 11457 (7832.7) | 12383 (8242.1) | 0.69 |
| (cell/mm$^3$) †1 | rectum | 19055 (13326.9) | 23416 (19798) | 0.37 |

Data are expressed as mean cell number (standard deviation: SD) per square millimeter.

†1 As for exceptions, those of ICC-CM and ICC-LM were expressed as mean cell number per cubic millimeter because of the thicknesses of the circular and longitudinal muscle involving ICC-CM and ICC-LM, respectively, differed by their location. *: $P < 0.05$, **: $P < 0.01$

### *III. Comparison of the distribution pattern and morphology of ICC-MP between the small intestine and the large intestine*

The subtype ICC-MP exists in both the small and large intestine, where it associates with the ENS. Comparison of ICC-MP present in the small intestine and the large intestine was done. The spatial relationship of both types of ICC with the myenteric plexus, can be seen in Fig. 3. The characteristic features between proximal and distal sites are also demonstrated in Fig. 5. Both ICC-MP showed a multipolar shape, with long branching processes that form an interconnecting network. ICC-MP of the small intestine, however, seemed to have thicker branching processes and more lateral branching than ICC-MP of the large intestine.

The cell density of ICC-MP of the large intestine was higher than that of the small intestine, with significant difference (Table 2). However, the c-Kit positive mean area of ICC-MP per unit area (/1mm$^2$) was larger in the small intestine than the large intestine (Table 2, Fig. 6A). Furthermore, the c-Kit positive mean area of ICC-MP in the proximal site was significantly larger than that in the distal site, for both the small and the large intestine (Table 2, Fig. 6B, C). For comparison, the c-Kit positive area of ICC-SMP did not show any difference between the proximal and distal sites in the large intestine (Table 2, Fig. 6D).

### *IV. Unique morphological feature of ICC-SMP*

Unique feature of ICC-SMP was found in this study. ICC-SMP had flat and dense network. The pattern of network was different between the proximal and distal sites. The connecting pattern of ICC-SMP in the proximal site was mesh-like (Fig. 7A) but that in the distal site was a ladder-

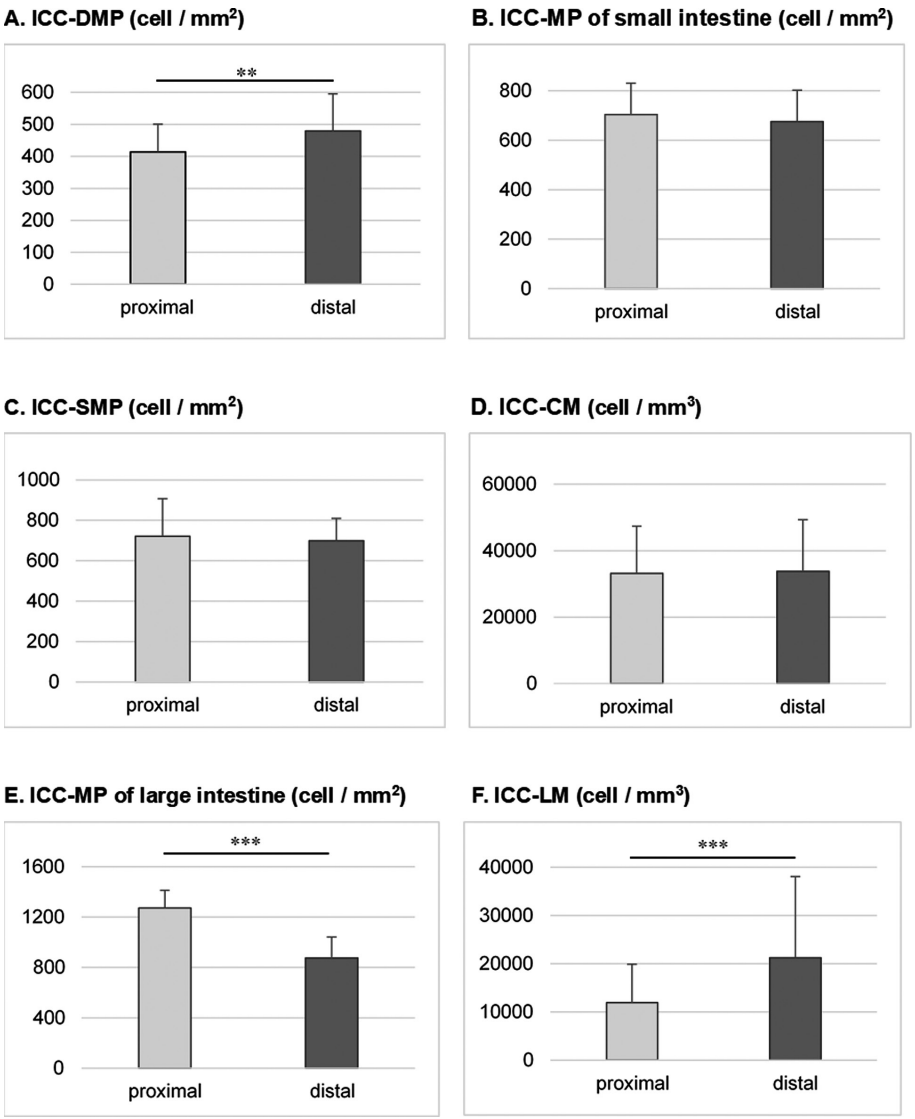


Fig. 4. Mean Area Densities of Subtypes of ICC. The total value for each item from Table 1 were visualized in graphs. The cell number per unit area (1 $mm^2$) were expressed on the Y axis (cells/$mm^2$). *$p < 0.05$, **$p < 0.01$

like pattern along with a circular muscle layer (Fig. 7B).

## Discussion

The aim of this study was to evaluate the distribution pattern of ICC subtypes to understand their role in gastrointestinal motility and interaction with the ENS, depending on their location. Based on the morphological analyses, using c-Kit and PGP9.5 to identify ICC and ENS, respectively, we measured the normal local density and morphological features of ICC and compared the number of ICC between mesenteric and anti-mesenteric sides, and in between proximal and distal sites using whole mount preparations of the mouse intestine. This approach enabled us to accurately evaluate the distribution density of ICC in the local area.

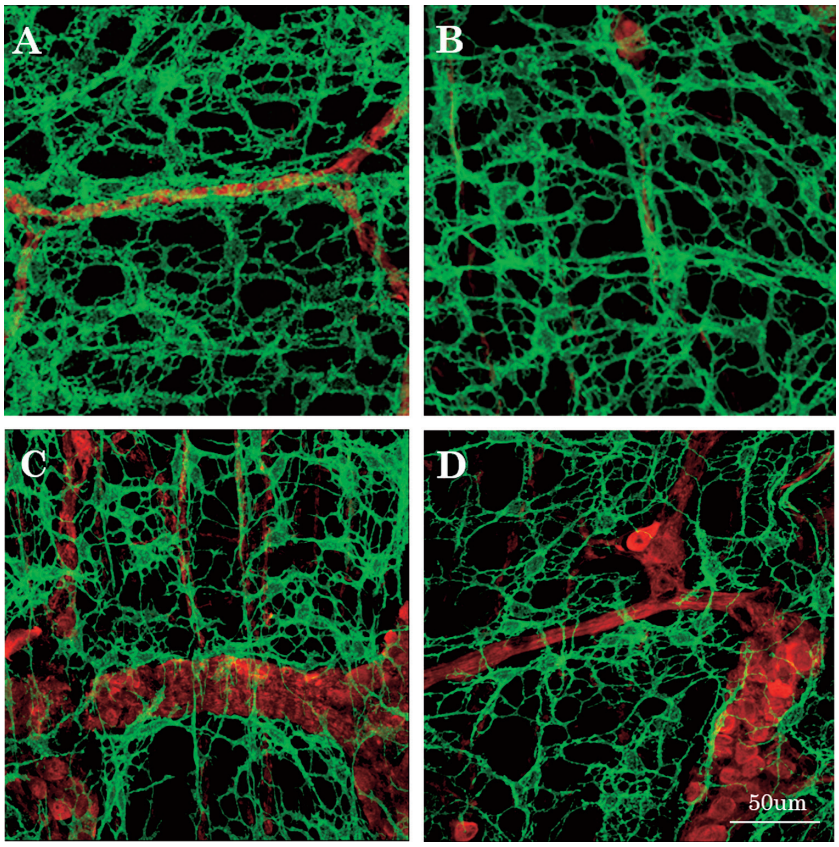


Fig. 5. Comparison of ICC-MP between the Proximal and Distal sites in the Small and Large Intestine. Photomicrographs show ICC-MP (green) and nerves (red). ICC-MP of small intestine (A, B) and the large intestine (C, D). A: jejunum, B: ileum, C: colon, D: rectum. Bar indicates 50 μm.

The major and novel findings of this study are presented below.

1) The distribution density of ICC varied depending on the site of the intestine. In general, there was no highly significant difference in cell density of most ICC subtypes between the mesenteric and the anti-mesenteric sides, except for ICC-MP in the rectum. In contrast, highly significant differences, in the distribution density, between proximal and distal sites were seen for ICC-DMP (distal > proximal) in the small intestine and ICC-MP (proximal > distal) and ICC-LM (distal > proximal) in the large intestine, respectively. Notably, the two major pacemakers—ICC-MP in the small intestine and ICC-SMP in the large intestine—showed no difference in cell density between the proximal and distal sites.

2) As a mutually existing subtype of ICC in both small and large intestines, ICC-MP of the large intestine had a significantly greater distribution density than those of the small intestine, although ICC-MP in the small intestine seemed to have denser network with much thicker processes and more branches than those of the large intestine. The predominance in c-Kit positive area of ICC-MP in the small intestine over those in the large intestine was also seen with a high significance. This indicates their morphologically denser network. Furthermore, in both the small and large intestine, larger c-Kit positive areas were seen in the proximal site than the distal site. In other words, there is a clear gradient of c-Kit positive area of ICC-MP in the intestine, which means that their network, including their processes and branches, is denser in the proximal site than in the distal site.

3) The network pattern of the processes of ICC-SMP in the

Table 2. Difference in cell density and c-kit positive area of ICC-MP between small intestine and large intestine

| | ICC subtypes | small intestine | | large intestine | | *p value* |
|---|---|---|---|---|---|---|
| **Cell Density**[†1] | ICC-MP (cell/mm$^2$) | 689 (126.4) | | 1074 (251.3) | | <0.0001** |
| **c-kit positive area per unit area (1 mm$^2$)**[†2] | ICC-MP (mm$^2$) | 0.374 (0.051) | | 0.287 (0.057) | | <0.0001** |
| | | proximal | distal | proximal | distal | |
| | ICC-MP (mm$^2$) | 0.384 (0.045) | 0.354 (0.052) | | | 0.004** |
| | | | | 0.323 (0.042) | 0.245 (0.039) | <0.0001** |
| | ICC-SMP (mm$^2$) | | | 0.270 (0.033) | 0.264 (0.028) | 0.33 |

[†1]: Data were expressed as mean cell number (standard deviation: SD) per square millimeter.

[†2]: Data were expressed as mean c-kit positive area of ICC (standard deviation: SD) per unit area (mm$^2$). The data of ICC-SMP are also presented for comparison. ***: P < 0.01*

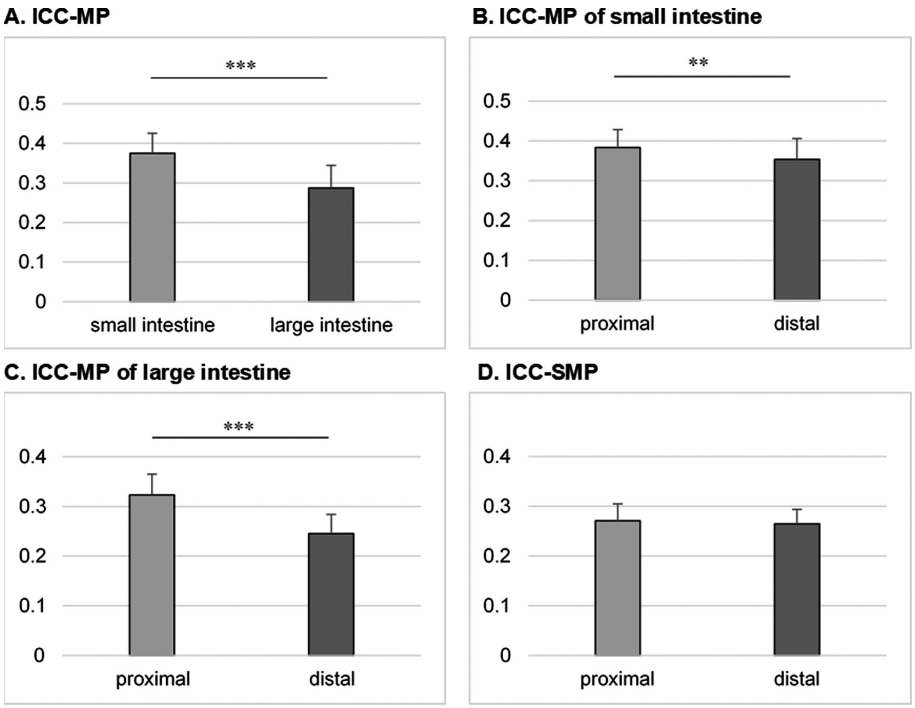


Fig. 6. Comparison of c-Kit Positive Areas (mm$^2$) Per Unit Area (1 mm$^2$). The data in Table 2 were visualized in graphs. c-Kit positive area of ICC-MP per unit area (1 mm$^2$) were expressed on the Y axis (mm$^2$). Note that ICC-MP of the small intestine occupied a larger area than those of the large intestine. The c-Kit positive area of ICC-SMP in the large intestine are also shown for comparison. ***p < 0.01*, ****p < 0.0001*

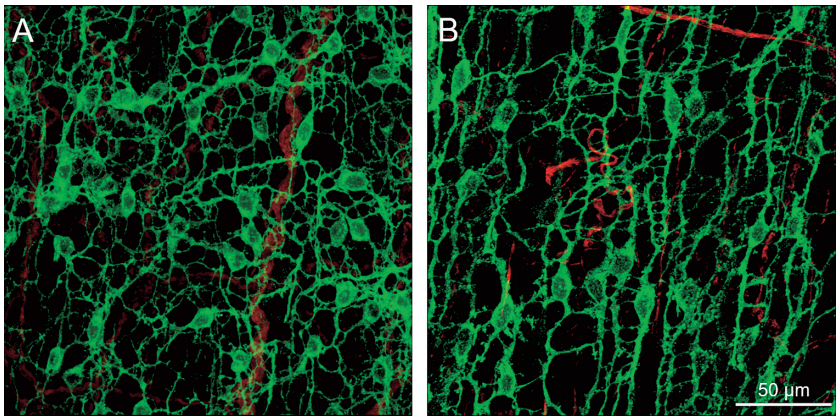


Fig. 7. Morphological Comparison of ICC-SMP between the Proximal and Distal Sites in the Large Intestine. Photomicrographs show ICC (green) and nerve cell elements (red) in the large intestine. ICC-SMP in the proximal site (A) and the distal site (B). ICC-SMP had a flat and dense network. The pattern of network was different in the proximal (colon) and distal sites (rectum). Note that the connecting pattern of proximal ICC-SMP (A) was mesh-like but distal ICC-SMP (B) was ladder-like circumferentially. Bar indicates 50 μm.

proximal site (colon) of the large intestine was mesh-like, whereas that in the distal site (rectum) was ladder-like. This is the first indication that the ICC-SMP network pattern, between the proximal and distal sites of the large intestine, is different.

The ICC play two major roles in gastrointestinal motility. One is that of a pacemaker and the other is as an intermediate for transmission between the ENS and the smooth muscles. ICC-MP in the small intestine and ICC-SMP in the large intestine have been known to act as pacemaker cells for intestinal motility (Barajas-Lopez and Huizinga 1989; Smith *et al.* 1987; Ward *et al.* 1994). ICC-MP in the small intestine generate pacemaker activity that develops into electrical slow waves (Huizinga *et al.* 1995; Ordog *et al.* 1999; Sanders *et al.* 2014; Ward *et al.* 1994). To the best of our knowledge, the present study is the first to compare the distribution density of ICC-MP between proximal and distal sites, and between mesenteric and anti-mesenteric sides of the murine small intestine. We found that there was no difference in the distribution density of ICC-MP between the proximal and distal sites and between mesenteric and anti-mesenteric sides, with any gradient. The mean distribution density of ICC-MP of small intestine was $689.2 \pm 20.3/mm^2$ (mean ± S.E.) in our study, that is close to the result ($725.3 \pm 20.2/mm^2$) of Mei et al. (2009). For over a century, it has been known that the frequency of slow waves and their contractions decrease from the duodenum to the ileum of the small intestine in rabbits (Alvarez 1914; Maslennikova 1961) and in humans (Christensen *et al.* 1966). The gradient was relative to the distribution density of ENS; therefore, the difference in gastrointestinal motility of a specific region of the intestine was speculated to come from the difference in the density of ENS (Maslennikova 1961). The result of the present study that there is constant distribution density of ICC-MP do not contradict the theory that ENS generates the gradient of contraction frequency in the small intestine. However, the fact that the c-Kit positive area of ICC-MP of the small intestine, was greater at the proximal site than at the distal site, indicates that their denser network in the proximal site could be involved in the gradient of slow wave. In contrast, ICC-SMP is important as a pacemaker that generates slow waves of the large intestine, as reported previously (Barajas-Lopez and Huizinga 1989; Plujà *et al.* 2001; Huizinga *et al.* 2011), and is less influenced by neural inputs (Alberti *et al.* 2005). High frequent contractions are constant all along the colon (Huizinga *et al.* 2011). In this study, we found that the density of ICC-SMP of the large intestine did not have a gradient in the proximal to the distal site and in the mesenteric to the anti-mesenteric side. Previous studies have also reported that ICC-SMP had no gradient in proximal, median, and distal sites of the large intestine in rats (Alberti *et al.* 2007) and mice (Wang *et al.* 2014). Furthermore, for

c-Kit positive area per unit area, there was no difference in the size of ICC-SMP between the proximal and distal sites. We consider that the quantitative feature that ICC-SMP has constant density, support the physiological feature that slow wave of the colon is constant in the full length of large intestine. Since high frequent contractions are less influenced by neural inputs, the density of ICC-SMP might be unrelated to the density of ENS, that is higher at the distal than the proximal site of the large intestine. In contrast, difference in morphological features of ICC-SMP between proximal and distal sites of the large intestine might be important. In the proximal site of the large intestine, the network pattern of the processes of ICC were mesh-like, whereas it was ladder-like in the distal site. However, the functional significance of the different network patterns of ICC-SMP in the large intestine is unclear.

For decades, propagating rhythmic motor patterns in the colon, identical to colonic migrating motor complexes, have been described as either neurogenic (blocked by tetrodotoxin) or myogenic (insensitive to tetrodotoxin) (Huizinga *et al.* 2011). Many studies have indicated the involvement of ICC-MP in the contraction of the large intestine (Alberti *et al.* 2007; Barajas-Lopez and Huizinga 1989; Bayguinov *et al.* 2010; Dickson *et al.* 2010; Liu *et al.* 1998; Plujà *et al.* 2001; Smith *et al.* 1987; Spencer *et al.* 2007). Recently, ICC-MP has been suggested to be the second pacemaker in the large intestine. Huizinga et al. (2011) proposed that there are two independent pacemakers in the rat colon, one housed in the ICC-SMP, independent of the ENS, and the other housed in the ICC-MP that is stimulus-dependent, usually dependent on neural activity *in vivo*. Both networks of ICC-SMP and ICC-MP may orchestrate propagating contractions as pacemaker cells in concert with ENS and have a significant role related to ENS. Previous studies have shown that ENS has a greater density in the large intestine than the small intestine. Our results showing a greater distribution density of ICC-MP in the large intestine than the small intestine, as with the density of ENS, is consistent with its important role. However, the fact that ICC-MP in the small intestine seemed to have denser network with much thicker processes and more branches than those of the large intestine might have some relation to the difference in the roles of ICC-MP between the small and large intestines. Further physiological investigation of ICC-MP is needed for the depolarization of the thick smooth muscle layer in the large intestine.

Smooth muscle cells, ICC, and platelet-derived-growth-factor-receptor-alpha positive cells make up a complex of electrically-coupled cells, known collectively as the SIP syncytium (Sanders *et al.* 2012; Sanders *et al.* 2014). Among all subtypes of ICC, intramuscular ICC (ICC-IM), namely ICC-CM and ICC-LM, in the large intestine are thought to be involved in neuro-effector transduction in GI muscles and have an important role in tonic inhibition. Our results showed that the distribution density of ICC-CM had no gradient between proximal and distal sites but had slight increase at the mesenteric side in the rectum. The average distribution density of ICC-LM was very low as the longitudinal muscle was very thin, but in the distal site (near the anal sphincter), the density of ICC-LM increased with the thickening of the longitudinal muscle. One of the features of ICC-IM was that there were regional dispersions, therefore when we examine the density of ICC-IM, we should check many spots from the preparations. In this study, we found that ICC-LM lie sparsely in the longitudinal muscle and nerve fibers almost never exist in whole mount preparations of the mouse intestine.

Previous studies have indicated that ICC-DMP are innervated by motor neurons and transduce part of the input from enteric motor neurons (Iino *et al.* 2004; Wang *et al.* 2003a; Ward *et al.* 2006). In the present study, there was no gradient of the quantity of nerve bundles on the deep muscular plexus between the proximal and distal sites by PGP9.5 positive area (data not shown). Contrary to the expectation, however, the distribution density of ICC-DMP was higher in the distal site of the small intestine but it did not show the same tendency as the density of ENS. Recently, ICC-DMP has received a great deal of attention in the generation of the segmentation pattern of the small intestine (Huizinga *et al.* 2011). In the distal site of the small intestine, ICC-DMP may facilitate the generation of the rhythmic segmentation motor pattern.

In this study, we found differences in the distribution density between mesenteric and anti-mesenteric sides in ICC-DMP in the jejunum and ICC-CM and ICC-MP in the rectum. Their functional meanings are not clear, although previous studies by Gabella et al. have revealed that ENS is distributed at a greater density in the mesenteric side than the anti-mesenteric side. The difference in the distribution of ICC may have some functional relevance to ENS.

Decreasing or abnormal ICC in the large intestine including irritable bowel syndrome (Eshraghian and Eshraghian 2011), Crohn's disease (Wang *et al.* 2007), hypertrophic pyloric stenosis (Huizinga *et al.* 2009), colonic cancer, diverticulitis (He *et al.* 2000), chronic pseudo-obstruction (Struijs *et al.* 2008), and delayed maturation of the ICC in a transient neonatal pseudo-obstruction (Kenny *et al.* 1998) has been demonstrated in various human gastrointestinal motility disorders. Moreover, there exists a discrepancy between reports of decreasing and unchanging of ICC in Hirschsprung disease, known as being caused by abnormal development of the ENS (Horisawa *et al.* 1998; Vanderwinden JM 1996; Yamataka *et al.* 1995). In addition, the possible involvement of other types of cells, such as fibroblasts, macrophages, or mast cells have been suggested for the regulation of smooth muscle movement (Komuro 2012). Therefore, the careful studies on the quantification and distribution of ICC, under normal conditions, are important for the diagnosis and treatment of clinical disorders related to ICC. Although the present study provides fundamental information on the topographical distribution and density of major subtypes of ICC in the mouse intestine, it would lend itself to the consideration of their normal functional and morphological knowledge about ICC in the gastrointestinal tract.

## Conclusion

In the present study, we measured the normal local distribution and density of ICC to compare the number of ICC between the mesenteric and anti-mesenteric sides and between the proximal and distal sites, using whole mount preparations of the mouse intestine. The distribution density of ICC varied depending on the site of the intestine. There were gradients of cell density in some subtypes, but the gradient tendency was not always proportional to other subtypes even in the same part of intestine. Namely, there were individual cell densities, morphological features, and network patterns of each subtype depending on the site of the intestine. Therefore, the careful studies on the quantification and distribution of ICC under normal conditions should be important in clinical applications for the diagnosis and treatment of disorders related to abnormal distribution of ICC.

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### Conflicts of Interest

The authors have no conflict of interest to declare.

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