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Distribution of interstitial cells of Cajal in *Meriones unguiculatus* and alterations in the development of incomplete intestinal obstruction

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Summary. The interstitial cells of Cajal (ICCs) act as pacemaker cells that are involved in gastrointestinal (GI) motility disorders, although the pathogenesis of these disorders is still unclear. The GI tract of Mongolian gerbils shares similar anatomical features with that of humans, but no investigation of ICCs has been reported in the GI tracts of this animal. In the present study, we first observed the distribution and morphological features of ICCs in the Mongolian gerbil GI tract. The ICCs were mainly distributed within the smooth muscle layers (ICC-IM), the myenteric plexus (ICC-MY), the deep muscular plexus in the small intestine (ICC-DMP) and the submucosal surface of the circular muscle layer in the colon (ICC-SM). The density of the ICC-IM gradually decreased from the stomach to the colon, whereas the density of the ICC-MY gradually increased. Second, we compared differences in the ICCs between the control and obstructed intestines, and no significant difference was observed in the number of ICCs after 7 days of obstruction. However, the numbers were reduced by approximately day 14 of obstruction. The pattern of immunoreactivity also partly differed from that of the control group, i.e., a scattered and interrupted network of ICCs was often observed. Western blotting revealed that p-Kit and SCF were significantly reduced in the dilated intestines by day 14. Our results indicate that the Mongolian gerbil may be a good animal model for studying changes in ICCs that may contribute to the pathogenesis of GI motility disorders.

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Introduction

The interstitial cells of Cajal (ICCs) are a group of specialized cells in the gastrointestinal (GI) tract that are divided into four subgroups based on their morphological features and locations in the gut (Rumessen and Vanderwinden, 2003; Belzer et al., 2004; Hanani et al., 2005; Sander and Ward, 2007; Iino et al., 2011): ICCs of the myenteric plexus (ICC-MY), ICCs of the intramuscular layer (ICC-IM), ICCs of the submucosa (ICC-SM) and ICCs in the deep muscular plexus (ICC-DMP). ICCs are attracting increasing attention because of their putative roles in the regulation of GI motility (Romert and Mikkelsen, 1998; Torihashi et al., 1999; Radenkovic et al., 2010; Akiho et al., 2011; Beyder and Farrugia, 2012). Because ICCs are characteristically arranged in close contact to the enteric nervous system and smooth muscle cells in the GI tract, they are considered as pacemakers that generate and propagate electrical slow waves in the alimentary tract (Takayama et al., 2002). In addition, they act as the mediators of input from the enteric nervous system to the GI smooth muscle cells (Wang et al., 2003). ICCs also serve as a stretch sensor in the GI tract.

Clinical studies have indicated that a large number of GI motility disorders, including inflammatory bowel disease, pseudo-obstruction, slow-transit constipation, achalasia, diabetic gastroenteropathy, small bowel atresia and Hirschsprung's disease (Dickens et al., 1999;

Vanderwinden et al., 2000; Schoenberg et al., 2002; Fintl et al., 2004, 2010; Lee et al., 2009; Tander et al., 2010) are common and frequently encountered diseases with different clinical manifestations. Although the pathogenesis of these disorders is still unclear, they show similar pathological changes, including a decrease of ICC numbers and the disruption of ICC networks. These findings indicate that ICCs are involved in the development of GI motility disorders. However, the underlying mechanism for ICC loss or impairment is not well known. Therefore, further investigation of ICCs may help in the development of a treatment for GI motility disorders.

Several animal models, including mice, rats, guinea pigs, and donkeys (Dickens et al., 1999; Vanderwinden et al., 2000; Lee et al., 2009), have been used in studies of the development and function of ICCs. Previous experiments have indicated that slow-wave disappearance is associated with either a reduction of ICCs and/or disruption of cellular networks in the GI tract in mice. Although mouse models have been useful for studying ICC functions, the mouse GI tract is somewhat different from humans morphologically, genetically and biochemically. Therefore, an animal model with a GI tract with similar microstructure and function to humans is necessary for further study of GI motility disorders.

Mongolian gerbils were developed as a small animal model for medical research more than 20 years ago. The current data indicate that Mongolian gerbils are valuable in a wide variety of research fields, including neurology, endocrinology, genetics, bacteriology, virology, oncology, pharmacology, toxicology and metabolic diseases (Fischer et al., 2011; Pires et al., 2011; Xu and Wang, 2011; Lv and Shi, 2012). The GI microstructure and function of Mongolian gerbils is thought to be similar to that of humans (Matsumoto et al., 1997). This animal model may be better than the mouse model for investigating human diseases.

The most important advance in ICC research came from the discovery that ICCs express the c-kit gene product (Huizinga et al., 1995), a proto-oncogene that encodes a transmembrane tyrosine kinase (KIT) and is allelic with the murine white spotting locus (W) (Kitamura et al., 2001). Inactivation of KIT in fetal and neonatal animals has often been used to evaluate the physiological functions of ICCs. Animals with mutations in the c-kit or steel gene, which encodes stem cell factor (SCF), the ligand for KIT (Ordög, 2008; Wang et al., 2008), are also used to study the physiological functions of ICCs. These reports indicate that the KIT/SCF signaling pathway is essential for the development, proliferation, differentiation and survival of ICCs. In the present study, we first examined the distribution of ICCs in the Mongolian gerbil GI tract and compared it with that of mice using immunohistochemistry with an anti-KIT antibody. Second, we evaluated the changes in ICCs in an incomplete intestinal obstruction model using immunohistochemistry and western blotting to further understand the pathogenesis of GI motility disorders.

Materials and methods

Animals and incomplete intestinal obstruction animal model

Twenty adult Mongolian gerbils (6-8 weeks old, weight 50-70 g, either sex) were purchased from the Animal Center of Capital Medical University (Beijing, China). They were divided into three groups as follows: normal group (5 mice), sham group (5 mice), incomplete intestinal obstruction group (7 and 14 days, 10 mice). All Mongolian gerbils were maintained in an environment under standard conditions, i.e., with a 12 hr light/dark cycle and free access to food and water.

In the normal control group, the whole GI tract of the Mongolian gerbils was immediately collected following euthanasia by an overdose of pentobarbital. In the incomplete intestinal obstruction group, after they were anaesthetized and surgically treated, a loop of the proximal colon was exposed and partially obstructed by the application of a silicon ring at the proximal 2cm (Won et al., 2006). In the sham group, the animals were also anaesthetized and underwent a surgical laparoscopy as above but without partial obstruction treatment. At 7 and 14 days after the operation, the distended segments of the small intestine and proximal colon were removed. All procedures were performed in accordance with the Health Guide for the Care and Use of Laboratory Animals of our university, and the protocol was approved by the Institutional Animal Care Committee.

Immunohistochemistry

The whole GI tracts of the Mongolian gerbils, including the stomach, distal small intestine 2 cm proximal to the ileocecal junction and the proximal colon were removed and the enteric contents were washed away with 0.01 M phosphate-buffered saline (PBS, pH 7.2). For cryosection, 5-7 mm pieces from each segment were embedded in an optimal cutting temperature compound (OCT) and quickly frozen with liquid nitrogen. Cryosections (10 μm thickness) were cut with a cryostat (Leica CM3050S) and collected on poly-L-lysine-coated glass slides. After fan drying, the sections were fixed with 100% acetone for 15 min (4°C) and then immunostained. To make whole-mount preparations, the GI tract was washed with PBS and then inflated with 100% acetone (30 min) at 4°C. After washing with PBS, the specimens were opened along the mesenteric border and the mucosa were removed with the aid of a dissecting microscope to separate the longitudinal muscle layer with the serosa containing the ICC-MY and the circular muscle layer containing the ICC-DMP in the small intestine or the ICC-SM in the colon.

For immunostaining, the specimens were washed twice (10 min each) with PBS containing 0.3% Triton X-

100 (PBST). After incubation in bovine serum albumin (BSA, 1% in PBS) for 30 min (25°C), the specimens were incubated with rat anti-KIT (Ack2, eBioscience; 1:100) antibody for 24 hrs (4°C). After washing with PBS, the specimens were incubated with the Cy3-labeled goat anti-rat IgG antibody (Zymed; 1:100) for 1 hr (25°C). Counter-staining was performed with DAPI (Molecular Probes) to detect nuclei, and the specimens were then mounted with Fluorescent Mounting Medium (Thermo Electron). The specimens were examined using a fluorescence microscope (Nikon 80i, Japan) or a TCS SP5 confocal laser scanning microscope (Leica, Germany) with an excitation wavelength appropriate for Cy3 (552 nm) or DAPI (461 nm). Control specimens were prepared in the same manner, but the first antibody was omitted.

Western blot analysis

For the western blot analysis, the musculature of the intestines of the sham and incomplete obstruction groups were prepared. The segments were dissected out sequentially at different distances (0-25 mm, 25-50 mm, 50-100 mm and >100 mm) from the the ileocecal junction. The tissue samples were homogenized in 20 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid, and 1 mM phenyl methyl sulfonyl fluoride. Homogenates were centrifuged at 10,000 x g for 30 min at 4°C, and the supernatants were collected. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 7.5% polyacrylamide gel and were transferred to a membrane (Immobilon-P, Millipore Co, USA). Membranes were blocked with 5% fat-free powdered milk in PBS containing 0.05% Tween-20 and then incubated with rabbit anti-p-KIT (Y721) antibody (1:1000), rabbit anti-SCF antibody (1:1000), and rabbit anti-,-actin antibody (1:1000). Proteins were visualized using a chemiluminescence system with HRP-conjugated anti-rabbit IgG (1:2000) (ECL Plus, Applygen Technologies Inc.).

Results

Distributions of ICCs in the Mongolian gerbil GI tract

Using immunohistochemistry, KIT-immunoreactive cells that were considered ICCs were distributed along the entire GI tract, including the stomach, small intestine and colon of the Mongolian gerbils (Fig. 1). In the fundus of the stomach, only one type of ICCs, ICC-IM, was observed in cryosections stained with Ack2 antibody. However, in the corpus and antrum of the stomach, in addition to ICC-IM, ICC-MY were encountered between the longitudinal and circular muscle layers. In the small intestine and colon, ICCs were often observed in three regions, i.e., ICC-DMP, ICC-IM and ICC-MY, and ICC-IM, ICC-MY and ICC-SM, respectively. There was no obvious difference between the distribution pattern of ICCs in Mongolian gerbils and mice (data not shown).

To show the shapes of the KIT-positive cells, we immunostained whole-mount preparations of the GI tract. ICC-IM were bipolar cells, with two long, thin processes that ran parallel to the long axis of the longitudinal or circular smooth muscle cells in the stomach (Fig. 2). The density gradually decreased from the fundus to the antrum. By contrast, ICC-MY possessed several thin extensions that formed a complete cellular network around the myenteric plexus between the circular and longitudinal muscle layers in the gastric corpus and antrum. Their density gradually increased from the corpus to the antrum.

In the small intestine, ICC-DMP and ICC-MY had multiple cellular extensions forming a well-defined network associated with the DMP or myenteric plexus, respectively. The ICC-IM were similar in shape and distribution pattern to those in the stomach, but their total numbers were fewer. They were often associated with nerve fiber bundles running parallel to the axis of the smooth muscle cells (Fig. 3). In the colon, ICCs were observed within the smooth muscle layers, around the myenteric plexus and along the submucosal surface of the circular muscle layer. ICC-IM and ICC-MY had a similar pattern to that observed in other segments of the gut, but the density gradually decreased from the small intestine to the colon. ICC-SM had several thin and long interconnected processes that formed a cellular network on the submucosal surface of the circular muscle layer (Fig. 3).

The distribution of ICCs in the Mongolian gerbil GI tract was summarized. Nine regions of each specimen (total 15 specimens) from the stomach, small intestine and colon (5 specimens/each organ) were examined, and the results are summarized in Table 1. We defined large numbers (+++) as 40 ICCs or more in an average of 9 fields, medium (++) as 20 to 39 ICCs, small (+) as less than 20 ICCs, and negative (-) as no ICCs observed.

Changes in ICCs in the incomplete intestinal obstruction model in Mongolian gerbils

In the incomplete obstruction group, an obvious distention of the GI tract was observed at 14 days after the operation. We investigated changes in the ICCs in Mongolian gerbils that had undergone incomplete obstruction. In the sham group, the ICCs in the small

Table 1. ICCs distribution in the GI tract.

	ICC-IM	ICC-DMP	ICC-MY	ICC-SM
Fundus	+++	_	_	_
Corpus	++	_	+	_
Antrum	++	_	++	_
Small intestine	++	+++	+++	_
Proximal Colon	++	_	+++	++
Distal Colon	+	_	+++	++

intestine had many continuous branches that connected with each other to form a completed cellular network. This pattern was consistent with the observations of the normal control animals in the current study. We noted no obvious change in the number and shape of ICCs at 7 days after the incomplete intestinal obstruction procedure. After 7 days, the number of ICCs gradually decreased, and it was more obvious in the dilated region near the ileocecal junction. Moreover, the remaining ICCs in the dilated small intestine were so scattered that it was difficult to form a completed cellular network (Fig. 4). Similar changes also occurred in the dilated proximal colon. By 14 days, a scattered and interrupted network of ICC-SM and ICC-MY was also observed in the proximal colon (data not shown).

A western blot analysis was also performed, and the results clearly showed a single immunoreactive band representing p-Kit at approximately 145 kDa and a single band indicating SCF at approximately 31 kDa in the intestinal musculature of the GI tract of the control group. In contrast, p-Kit and SCF were significantly reduced in the dilated small intestinal musculature at 14

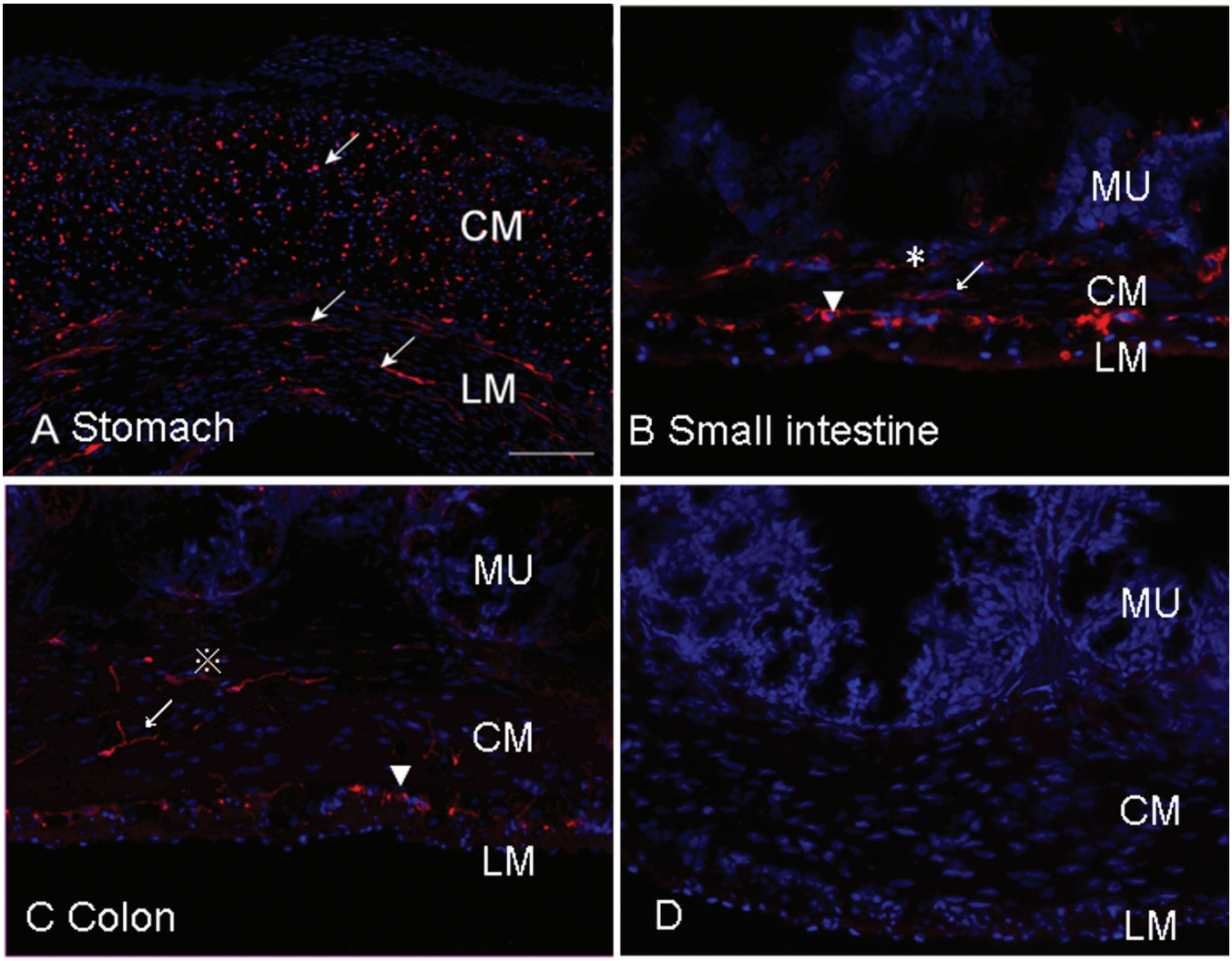

Fig. 1. Immunofluorescence photographs obtained from cryosections showing the distribution of ICCs in the gastrointestinal tract of the Mongolian gerbils. c-Kit-immunoreactive cells (red color), indicating ICCs in the stomach, were observed within the circular and longitudinal muscle layers (arrows) **(A).** In the small intestine, ICCs were widely distributed and were associated with the deep muscular plexus (*) and located around the myenteric plexus region (arrowhead) and within the circular and longitudinal muscle layers (arrows) **(B)**. In the colon, c-Kit-immunoreactive cells were observed around the myenteric plexus region (arrowhead), along the submucosal surface of the circular muscle layer (※) and within the circular (arrows) and longitudinal muscle layers **(C)**. No positive cells were observed in the negative control sections in which the anti-c-Kit antibody was omitted **(D)**. The nuclei were stained with DAPI (blue). MU mucosa; CM circular muscle layer; LM longitudinal muscle layer. arrow: ICC-IM; arrowhead: ICC-MY; *ICC-DMP; ※ICC-SM. Scale bar 50 μm.

days after obstruction (Fig. 5).

Discussion

Mongolian gerbils had been introduced as a suitable animal model for GI pathogenesis experiments and exhibited a more severe *Helicobacter pylori* (*H. pylori*) infection and evident histological changes of the stomach mucosa compared to other established animal models, e.g., mice, dogs, pigs. These data indicate that the Mongolian gerbil has a greater inflammatory reaction than other animal models and is more similar to humans (Matsumoto et al., 1997). This finding encouraged us to study the fine structure of the GI tract

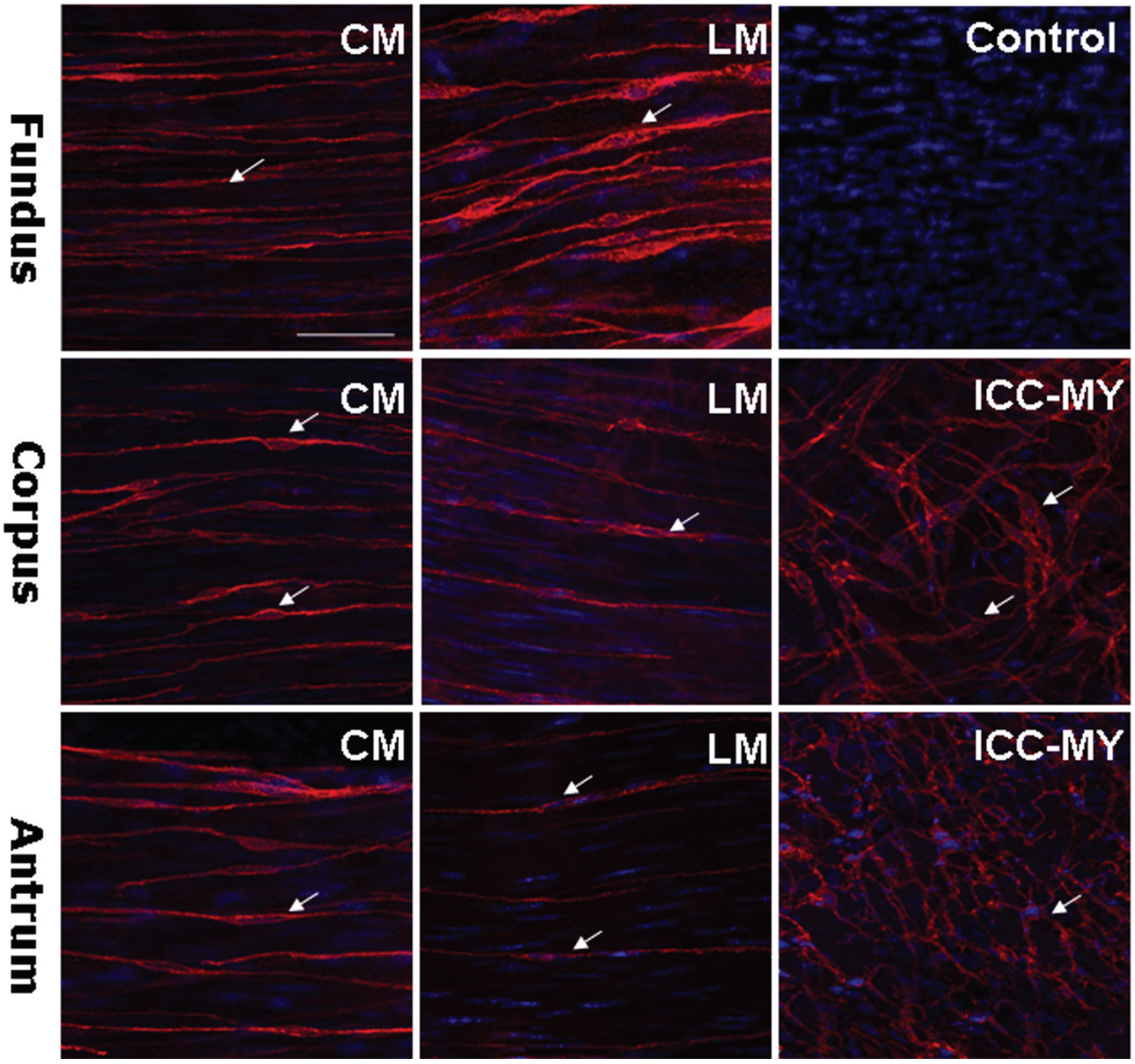

Fig. 2. Whole mount preparations stained with anti-c-Kit antibody showing shapes of ICCs (red) in the stomachs of the Mongolian gerbils. ICC-IM were bipolar cells with long processes (arrows) that ran parallel to the long axis of the circular (CM) and longitudinal (LM) muscle cells. Their density gradually decreased from the fundus to the antrum within the muscle layers, while the ICC-MY possessed multiple thin extensions that formed an intact cellular network around the myenteric plexus in the corpus and antrum. The number of ICC-MY in the antrum was greater than that of the corpus. No positive staining was observed in the negative control. The nuclei were stained by DAPI (blue). Scale bar: 50 μm.

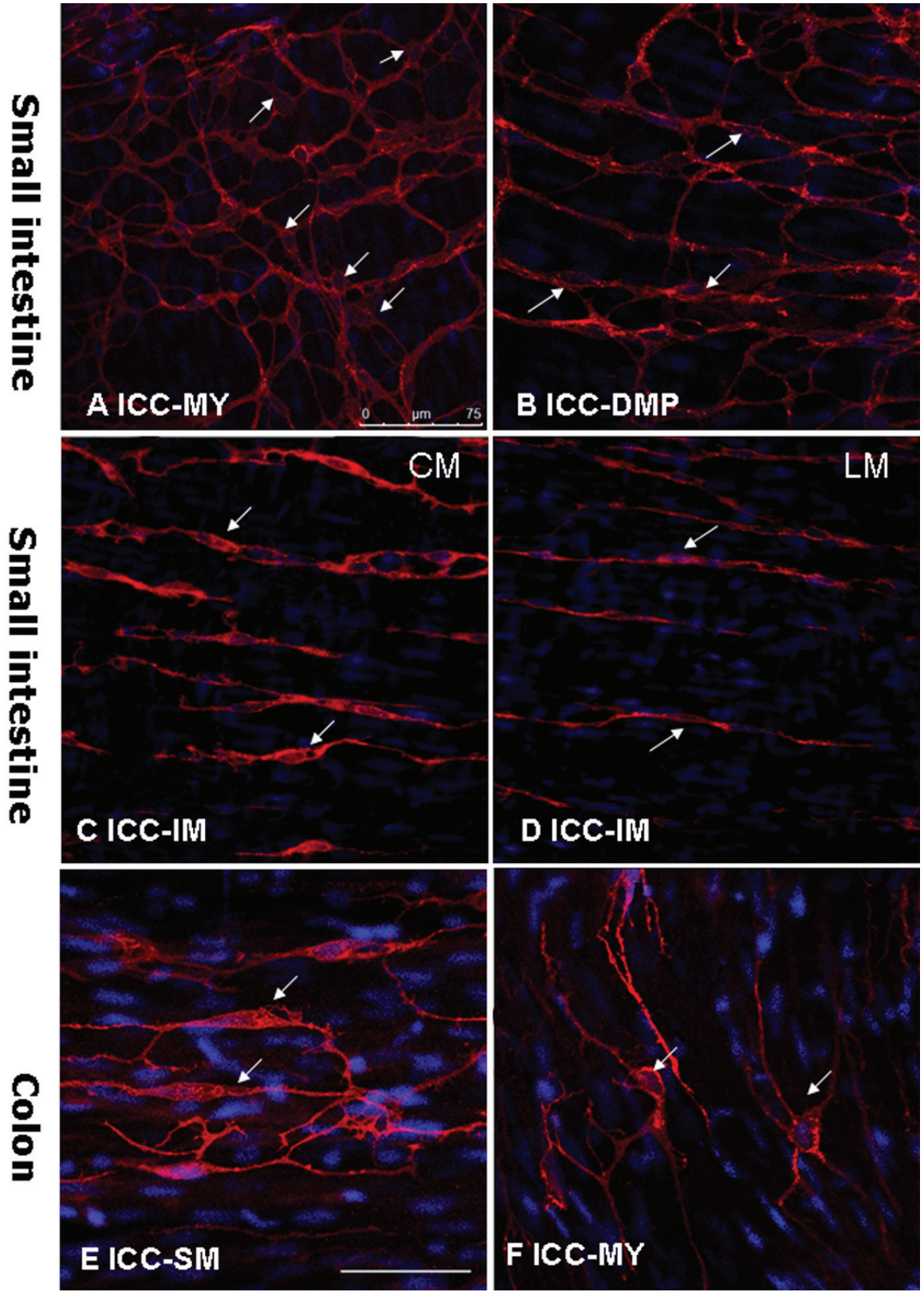

Fig. 3. Whole mount preparations showing ICCs in the small intestine and colon. The ICC-MY had highly branching processes (arrows) and formed a well-defined cellular network around the myenteric plexus region **(A).** The ICC-DMP were also observed with a number of thin and long processes that were interconnected to form a cellular network **(B)**. The ICC-IM within the circular **(C)** and longitudinal **(D)** muscle layers displayed bipolar processes that ran parallel to the long axis of the muscle fibers. The ICC-SM **(E)** and ICC-MY (F) in the colon possessed several thin processes that formed an intact cellular network. The nuclei were stained with DAPI (blue). CM circular muscle layer; LM longitudinal muscle layer. Scale bars: A-D, 75 μm; E, F, 25 μm.

of Mongolian gerbils to further understand the pathogenesis of GI motility disorders. We demonstrated that the lengths of the small intestine and colon in Mongolian gerbils were approximately 30 cm and 10 cm, respectively, and that their histological features were similar to other animals. Moreover, the Mongolian gerbil is larger than the mouse in size and is a suitable animal disease model.

ICCs have been suggested to be a regulator of GI motility from the esophagus to the anus in animals (Hagger et al., 1998; Torihashi et al., 1999). The current study demonstrated that ICCs in Mongolian gerbils were distributed throughout the GI tract, including the stomach, small intestine and colon, with some regional

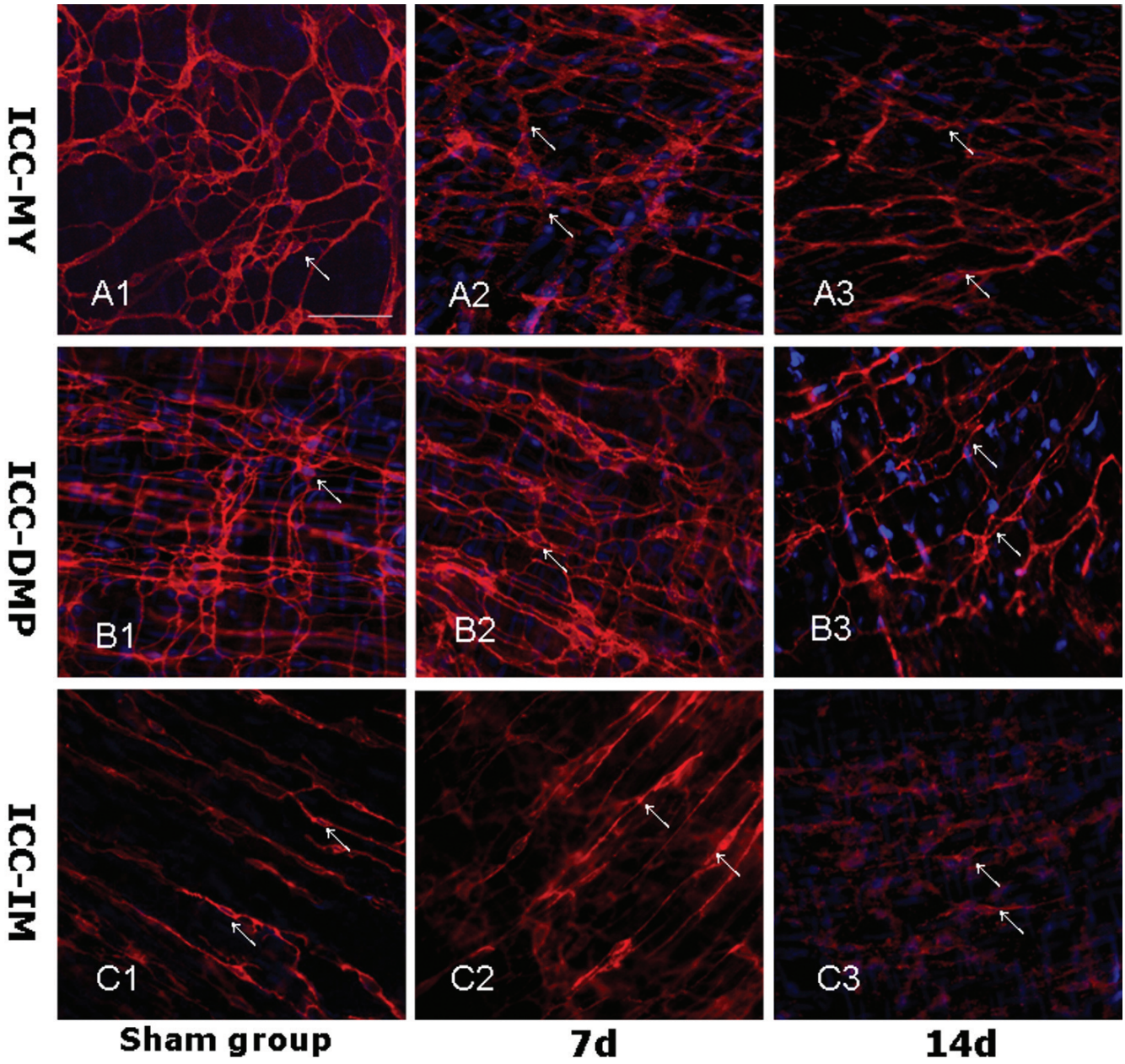

Fig. 4. Whole mount preparations showing ICCs in the dilated small intestine of the incomplete obstruction groups. The distribution of the ICCs in the sham group was similar to that in normal animals, i.e., ICC-MY **(A)**, ICC-DMP **(B)** and ICC-IM **(C)**. No significant difference was found in the number or staining density of ICCs at 7 days after the incomplete obstruction was created (A2, B2, C2) compared with the control group. However, the ICC-MY, ICC-DMP and ICC-IM were clearly reduced in number and density, and their networks were interrupted after 14 days (A3, B3, C3). The nuclei were stained with DAPI. Scale bar: 25 μm.

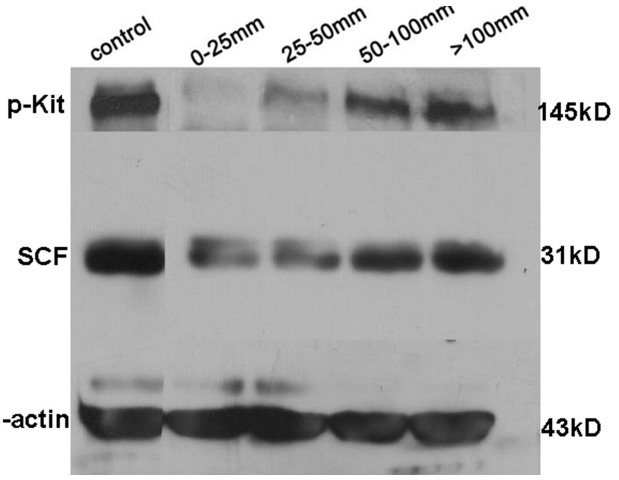

Fig. 5 Western blot analysis of the expression of p-Kit and SCF in the small intestinal muscle tissues of the sham and incomplete intestinal obstruction groups. p-Kit and SCF were significantly reduced in the dilated intestinal musculatures at 14 days after obstruction. The closer to the obstruction site, the more prominent the change. ß-actin expression was used as a loading control for p-Kit and SCF.

differences. Our results showed that the distribution patterns and morphologies of ICCs in the Mongolian gerbils were similar to those of other animals, such as mice, rats, guinea pigs and humans (Radenkovic et al., 2010). This similarity suggests that Mongolian gerbils can be used to study ICC-related diseases.

We created an incomplete mechanical intestinal obstruction in Mongolian gerbils to investigate changes in the ICC population that occurred in GI disorders. After 7 days, the number of ICCs gradually reduced, and it was markedly decreased in the distended segments by 14 days after obstruction. These changes suggest that ICCs are involved in the development of GI motility disorders. Of the ICCs, ICC-MY and ICC-SM work as pacemaker cells to trigger the generation of slow waves in the tunica muscularis of the small intestine and colon (Kito, 2011). ICC-IM are innervated preferentially by enteric motor nerves, and they act as the mediators of input from the enteric nervous system to GI smooth muscles, mediating excitatory and inhibitory neurotransmission. Thereby, changes in the number and distribution pattern of ICCs during the development of the intestinal obstruction may alter their functional responsibility in the GI tract. The incomplete mechanical intestinal obstruction was produced by applying a silicone ring, and the ICC decrease caused by the dilated intestine may reduce GI motility and accelerate obstruction development. In fact, a number of papers have demonstrated that the loss of ICCs or the blockade of the KIT signaling pathway leads to GI motility dysfunction. However, the mechanism of reducing the number of ICCs is still unclear.

It has been demonstrated that the KIT signaling pathway is required for the maintenance and survival of mature ICCs in the GI tract (Takayama et al., 2002). The inhibition of the KIT signaling pathway decreased the number of ICCs and inhibited ICC proliferation in adult guinea pigs (Mei et al., 2009). Mutations at the W locus caused variable decreases in KIT activity, which in turn affected the number of ICCs and disrupted their cellular networks in the GI tract. However, the same phenomenon was also observed in Sl/Sld mutant mice (SCF defect). SCF, encoded by the steel gene, is primarily expressed in smooth muscle cells and neurons in the GI tract. Reducing SCF in intestinal muscle has been related to the development of GI motility disorders (Mei et al., 2006). In the present study, the number of ICCs was reduced and their cellular networks were disrupted after the obstruction was created. We counted the number of ICC-MYs in a 5 mm length of dilated small intestine at 14 days to exclude the influence of intestinal dilatation. The absolute number of ICC-MY was clearly reduced. Meanwhile, SCF and p-Kit activity were significantly reduced in the obstructed intestines by 14 days, indicating that activation of the KIT/SCF signaling pathway was also required for the maintenance and survival of ICCs in the Mongolian gerbils. The disappearance of ICCs following KIT/SCF signaling pathway inhibition raises an important question as to the fate of ICCs after intestinal obstruction. We considered that the ICCs might transdifferentiate to a type of alpha-SMA-positive cells, perhaps a phenotype of smooth muscle cells, when a loss-of-function of Kit occurs. Torihashi and colleagues also reported similar findings in the small intestines of neonatal mice treated with a Kit-neutralizing antibody (Torihashi et al., 1999). Therefore, we proposed that intestinal obstruction caused the intestinal expansion with hypertrophy of smooth muscle cells leading to the reduction of SCF expression. Insufficient SCF will reduce the activity of the KIT/SCF signaling pathway and lead to a decrease in the number of ICCs. However, the precise mechanism behind the ICC decrease requires further study.

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Conflicts of interest We declare no conflicts of interest.

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