Loera-Valencia, R., Wang, X., Wright, G.W.J. *et al.* *Ano1* is a better marker than *c-Kit* for transcript analysis of single interstitial cells of Cajal in culture. *Cell Mol Biol Lett* 19, 601–610 (2014).

https://doi.org/10.2478/s11658-014-0214-4

CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 23 April 2014
Final form accepted: 15 October 2014

Volume 19 (2014) pp 601-610
DOI: 10.2478/s11658-014-0214-4

Short communication

# *Ano1* IS A BETTER MARKER THAN *c-Kit* FOR TRANSCRIPT ANALYSIS OF SINGLE INTERSTITIAL CELLS OF CAJAL IN CULTURE

RAÚL LOERA-VALENCIA[1, 2,]*, XUAN-YU WANG[2], GEORGE W.J. WRIGHT[2], CARLOS BARAJAS-LÓPEZ[1] and JAN D. HUIZINGA[2]
[1]División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa San José 2055, Col. Lomas 4a Sección, C.P.78216 San Luis Potosí, SLP, México, [2]Farncombe Family Digestive Health Research Institute, Department of Medicine, McMaster University, HSC-3N8, 1200 Main Street West, Hamilton, ON, L8N 3Z5, Canada

**Abstract:** The interstitial cells of Cajal (ICC) drive the slow wave-associated contractions in the small intestine. A commonly used marker for these cells is *c-Kit*, but another marker named *Ano1* was recently described. This study uses single-cell RT-PCR, qPCR and immunohistochemistry to determine if *Ano1* could be reliably used as a molecular marker for ICC in single-cell mRNA analysis. Here, we report on the relationship between the expression of *c-Kit* and *Ano1* in single ICC in culture. We observed that *Ano1* is expressed in more than 60% of the collected cells, whereas *c-Kit* is found only in 22% of the cells (n = 18). When we stained ICC primary cultures for c-KIT and ANO1 protein, we found complete co-localization in all the preparations. We propose that this difference is due to the regulation of *c-Kit* mRNA in culture. This regulation gives rise to low levels of its transcript, while *Ano1* is expressed more prominently in culture on day 4. We also propose that *Ano1* is more suitable for single-cell expression analysis as a marker for cell identity than *c-Kit* at the mRNA level. We hope this evidence will help to validate and increase the success of future studies characterizing single ICC expression patterns.

**Keywords:** Interstitial cells of Cajal, *c-Kit*; *Ano1,* Multiplexed RT-PCR, Single-cell PCR, Transcriptional regulation, ICC marker, Small intestine, Primary cultures, Pacemaker cells

---

* Author for correspondence. Email: raul.loera@ipicyt.edu.mx; phone: +52 444 834 2000 x2033; fax: +52 444 834 2010

Abbreviations used: *Ano1* – anoctamin 1, HS – HEPES buffer saline solution, ICC – interstitial cells of Cajal, NC – no cell control

## INTRODUCTION

Pacemaker cells called the interstitial cells of Cajal (ICC) drive the slow wave-associated contractions in the small intestine [1–3]. Research on the physiology and biochemistry of these cells through patch clamping and other electrophysiological techniques helped to gain insight into their function as the pacemakers of the gut [4–7]. However, other molecular techniques, such as RT-PCR and qPCR have only been used in a limited way because there is no pure culture of ICC or ICC cell line on which to perform gene expression analysis separately from the associated tissues, such as the enteric nerves and smooth muscle [8]. To date, only one transcriptomic analysis of ICC has been achieved after enrichment and purification of cell samples through fluorescence-activated cell sorting [9], but the implementation and validation of such a method requires specialized equipment and considerable economic investment. With the availability of transgenic mice with copGFP-expressing ICC [10], primary cultures have been used to improve identification in electrophysiological or immunohistochemical analyses [11, 12] but not in single cell characterization.

One alternative for the molecular analysis of ICC is the single-cell RT-PCR technique, which allows the collection of individual cells from a mixed culture [13]. The use of single-cell RT-PCR has increased in recent years thanks to the introduction of new technologies and the implementation of ready-to-use PCR products. However, the research on single cell expression profiling with ICC has been limited, with only few publications on the subject, mostly dedicated to the identification of the ICC through *c-Kit* expression, which is a broadly accepted ICC marker [1, 3, 14, 15]. Some of the problems encountered performing single-cell RT-PCR with ICC are the small amount of genetic material obtained and the need to amplify the ICC marker *c-Kit* from every sample, since the most simple form of the protocol allows the amplification of only one target [16].

The large majority of experiments in ICC have been performed in culture, and previous reports have indicated that culture conditions may affect the biochemistry and function of ICC [17, 18]. In particular, *c-Kit* is a gene that can be greatly influenced by the presence of serum factors like TGF-beta, which significantly decreases the half-life of *c-Kit* mRNA [19]. This phenomenon could affect the results of expression studies involving single-cell RT-PCR and using *c-Kit* as a molecular marker. Recent evidence has identified the calcium-activated chloride channel TMEM16A/anoctamin 1 (*Ano1*) in ICC [10]. Immunofluorescence studies reported 100% co-localization of this channel with *c-Kit*, and it is now accepted as an additional marker of ICC identity in both culture and tissue [20, 21].

## MATERIALS AND METHODS

### ICC primary cell culture

Short-term primary cultures of ICC were generated by enzymatic digestion of dissected small intestinal muscle tissue as previously described [22, 23]. Small intestines were removed from 5- to 15-day old CD-1 mice (Charles River Laboratories) and dissected using blunt dissection. The gut wall was cut open at the mesenteric border, and then the mucosa was removed along with the mesentery. The muscle was cut into pieces and incubated for 15 min at 36ºC in HEPES-buffered saline (HS) with the addition of 1 mg/ml type F collagenase, 1 mg/ml bovine serum albumin, 0.5 mg/ml papain, 0.5 mg/ml soybean trypsin inhibitor and 0.2 mg/ml (−)-1,4-dithio-L-threitol (all from Sigma). After trituration of the smooth muscle, the cell suspension was settled on collagen-coated cover slips and cultured for 3–4 days before use, using the Clonetics SmGM-2 system (Lomax, supplied by Cedarlane).

All of the procedures were carried out in accordance with regulations from the Animal Research Ethics Board (AREB) of McMaster University in accordance with guidelines from the Canadian Council on Animal Care.

### Relative expression RT-PCR

To assess the relative expression of *c-Kit* and *Ano1* compared to GAPDH, primary cultures of ICC were prepared as described above. Day 0 corresponded to a stabilized culture in serum-free solution, while days 2 and 4 correspond to that many days of culture in the normal culture medium. The Ambion Cells to cDNA II Kit for cDNA extraction was used according to the manufacturer's instructions. The internal primers designed for *c-Kit* and *Ano1* are also qPCR compatible. We detected GAPDH expression with the primers GAPDHF 5'-CCATGGAGAAGGCCGGGG and GAPDHR 5'-CAAAGTTGTCATGGATGACC (PCR product: 198 bp). The program included an initial denaturing of 95ºC for 5 min, followed by 35 cycles of 10 s of denaturation at 95ºC and annealing/extension at 60ºC for 5 s. A melting curve was applied to ensure the specificity of the PCR products (65 to 95ºC with 0.5ºC steps every 5 s).

### Single cell isolation and RNA extraction

In order to isolate the ICC in primary culture for RT-PCR, we utilized a patch clamp rig as described elsewhere [23]. ICC-MP were identified by their roughly triangular shape with a process at each apex, found singly. Protease (0.1 mg/ml) was used to detach ICC from the collagen-coated coverslips. Unpolished, low-resistance pipettes were used to remove cells from the coverslips. Cells were removed by applying negative pressure to the pipette. A no cell (NC) control was included. For it, we simulated the collection of a cell by lowering the pipette into the bath solution. The pipettes for single cell extraction contained 0.6 μl of RNase-free 10x RT Buffer with RNase inhibitor (20 units per sample) to a final volume of 6 μl. The contents of the pipette were expelled with positive pressure into a PCR tube containing 12.5 μl of RNase-free RT mixture consisting of 2.3 μM oligo (dT), 150 μM dNTPs, 1.2 mM dTT, 3.6 mM $MgCl_2$ and 1.4 μl of 10x RT Buffer (Life Technologies) along with 0.5 μl of 1% NP40 detergent to cause cell membrane disruption.

The reaction was incubated at 65ºC for 2 min. After the addition of 1 µl reverse transcriptase (Superscript III, Invitrogen), the sample was placed at 50ºC for 90 min. For positive controls, tissue extracted from adult CD-1 murine brains was triturated in a mortar with a pestle in liquid nitrogen. Afterwards, we weighed 10–20 mg of tissue and collected it in Eppendorf tubes with 500 µl of lysis solution from an RNeasy RNA isolation kit (Qiagen). The RNA was obtained from the lysis solution using an affinity column and was collected for cDNA synthesis using the instructions of the Superscript III First Strand Synthesis Kit (Invitrogen).

### Single-cell RT-PCR

The single cells obtained from primary cultures were tested for *Ano1* and *c-Kit* expression using a nested approach. The external primers for pre-amplification were:

Ano1F 5'-TGTACTTTGCCTGGCTTGGAGC and Ano1R 5'-CACCTGGC AATGCAGCCGTA (PCR product: 700 bp); and c-kitF 5'-GCTCAT TGGCTTTGTGGTTGCAG and c-kitR 5'-ATGCGCCAAGCAGGTTCACAA (PCR product: 404 bp).

For nested PCR we used the internal primers:

Ano1intF 5'-CAACTACCGATGGGACCTCAC and Ano1intR 5'-AATAGG CTGGGAATCGGTCC (PCR product: 170 bp); and c-kitintF 5'-ATA GACCCGACGCAACTTCCT and c-kitintR 5'-AACTGTCATGGCAGCATC CGAC (PCR product: 150 bp).

Pre-amplification of the targets was carried out on half of the single cell cDNA, or 200 ng of tissue cDNA, by cycling 30 times at 50ºC and extending for 1 min at 72ºC. Then, nested PCR was carried out using internal specific primers. The PCR protocol was performed on a CFX96 thermal cycler (Bio-Rad Laboratories Canada Ltd.): initial denaturation for 3 min at 94ºC, then 35 amplification rounds of denaturation for 15 s at 94ºC, alignment for 15 s at 55–58ºC, and extension for 30 s at 72ºC. The final extension was 5 min at 72ºC.

For both amplifications, recombinant Taq Polymerase was used according to the manufacturer's instructions (Life Technologies). Negative controls were performed without a template; no false amplifications were obtained. The resulting products were analyzed via agarose electrophoresis in 1.5% agarose gels (Invitrogen) stained with 1 µg/ml ethidium bromide (Sigma-Aldrich). Images were obtained with a Gel-Doc 2000 documentation system (Bio-Rad Laboratories Canada Ltd.). The identities of all of the amplicons produced were confirmed by sequencing (MOBIX Laboratories, McMaster University).

### ANO1 and c-KIT immunohistochemistry

For immunohistochemistry, both musculature whole-mount tissue and cultured cells were made from the proximal jejunum of CD1 mice processed according to the following protocol. Tissues were fixed in ice-cold acetone for 10 min. After incubation with 5% normal goat serum for 1 h to block non-specific staining, tissues were incubated with monoclonal rat anti-*c-Kit* (ACK4, 1:200, Cedarlane) overnight, followed by Cy3 conjugated goat anti-rat IgG (1:600, Jackson ImmunoResearch) incubation for 1 h at room temperature. After c-Kit staining, the tissues were fixed again with 4% (w/w) paraformaldehyde in phosphate-buffered saline (PBS) for 1 h. The tissues were incubated with rabbit anti-ANO1 (1:100, AbCam Inc.) and then with Alexa 488-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch). All of the antibodies were diluted in 0.3% Triton X-100 in PBS (pH 7.4). Control tissues were prepared by omitting primary antibodies. Pictures were taken using a confocal microscope (Zeiss LSM 510) with excitation wavelengths (543 nm and 488 nm) appropriate for Cy3 and Alexa 488.

## RESULTS AND DISCUSSION

### *Ano1* and *c-Kit* relative expression

In our relative expression analysis, we observed that *c-Kit* levels remain constant during primary culture, but they were low on day 4 compared to *Ano1*, which increased around fourfold ($p < 0.05$, Fig. 1A). This suggests that Ano1 is a better candidate for ICC identification in single cell expression analyses. The effect of serum on *c-Kit* mRNA regulation has been shown in other cell types [19], but the precise dynamics of *c-Kit* transcription, translation and *cys*-acting mechanisms in ICC require further investigation.

### Standard single-cell *Ano1* and *c-Kit* RT-PCR

Since Ano1 levels were highest on day 4, we looked for Ano1 and c-Kit expression in single ICC at this point during culture. We were able to amplify two genes from single interstitial cells of Cajal: *Ano1* and *c-Kit*. We found that of the eight *Ano1*-positive cells, only one exhibited *c-Kit* expression (Fig. 1B). Of 11 cells tested, 3 cells did not exhibit *Ano1* or *c-Kit* amplification. These cells were not taken into account in the percentage reported because we cannot be sure that any PCR product could be amplified from their cDNA.

### Multiplexed RT-PCR and immunohistochemistry

The possibility of a multiplexed approach to increase the number of targets amplified has been reported elsewhere [24]. We applied this methodology to single ICC for this study. We revisited the ratio of *Ano1* and/or *c-Kit* expression in these multiplexed experiments with similar results ($n = 18$ cells; 12 *Ano1*-positive cells; 4 *c-Kit*-positive cells). Additional genes were amplified from the *Ano1*-positive cells (voltage-gated porins and potassium channels, data not shown), confirming that cDNA had been synthesized correctly and the lack of *c-Kit* expression was not an artifact of the technique used.

At the protein level we found 100% co-localization between *Ano1* and *c-Kit* in both tissue and cultured cells (Fig. 2), which is consistent with previous reports. This suggests that our negative *c-Kit* results could be the product of the low levels of *c-Kit* mRNA present in the cells at the moment of the extraction, a diminished half-life of its mRNA, or a combination of the two factors, whereas the *Ano1* transcript levels increased.

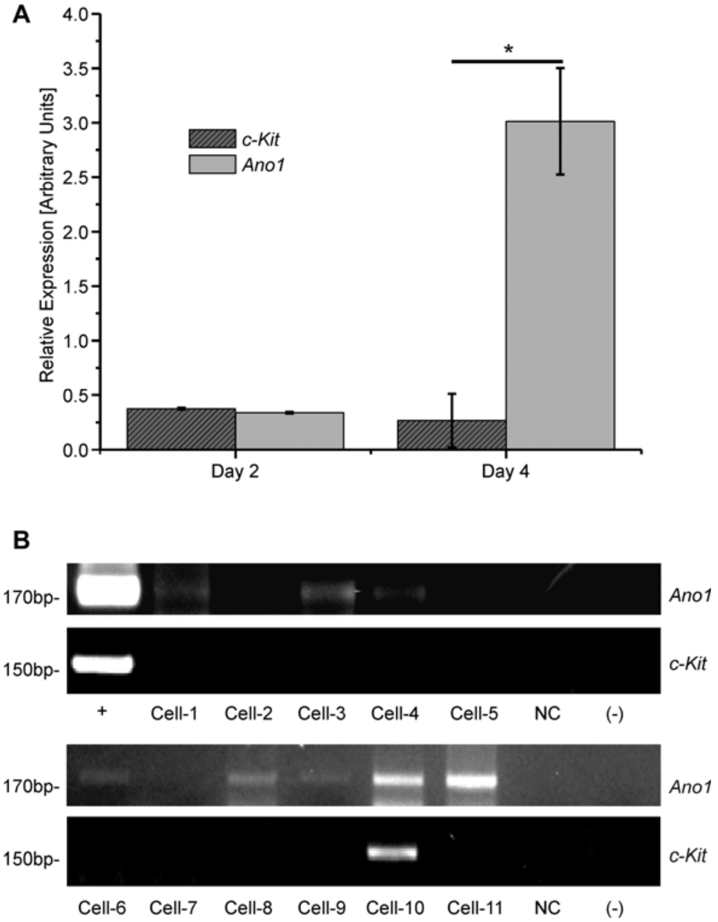

Fig. 1. *Ano1* abundance over *c-Kit* in single isolated interstitial cells of Cajal (ICC). A – Relative expression quantification of *Ano1* and *c-Kit* transcripts from whole small intestinal ICC primary cultures. The data was normalized to the level on day 0 of culture. The asterisk indicates statistical significance ($n = 3$ per group; $p < 0.05$) between the expression of *Ano1* and *c-Kit* in primary ICC cultures on day 4. B – *Ano1* and *c-Kit* RT-PCR from single ICC in culture. Whole intestine cDNA (0.2 µg) was used as a positive control. Every column represents the PCR products obtained from a single ICC cDNA. NC denotes a no cell control, while the negative control was performed without template. The identity of the products was confirmed by sequencing.

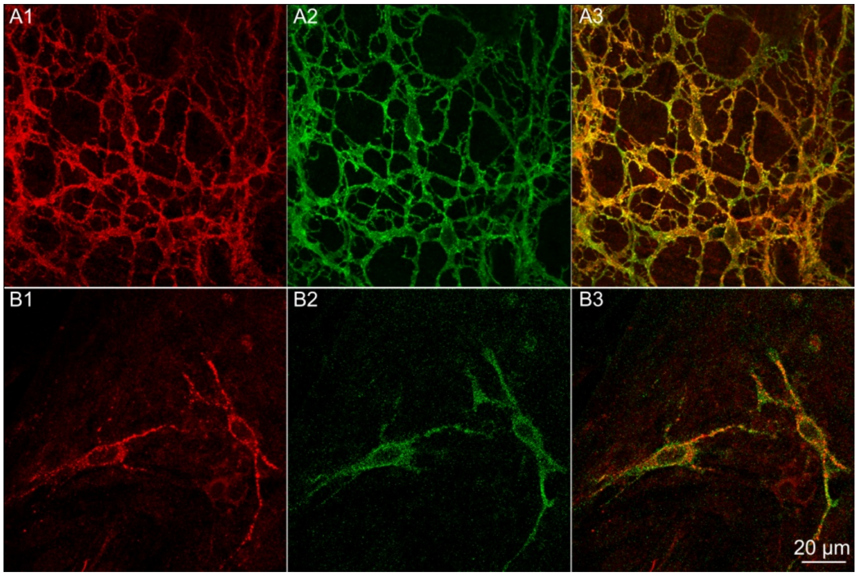

Fig. 2. c-Kit (red) and ANO1 (green) immunoreactivities in mouse jejunum musculature. A1 through A3 – Wholemount preparations show a dense ICC-MP network. B1 through B3 – Cultured preparations show triangle or multipolar-shaped ICC. Co-localization of c-Kit and ANO1 was 100% in ICC-MP of both tissue (A3) and cultured cells (B3).

While *Ano1* transcript levels in cultures seem to increase by day 4, it is likely that they reach a steady-state level. This mainly because Ano1 is required for the generation of slow waves [25] and also because the recorded slow waves maintain their characteristics stably after several days in culture [26].

ANO1 has been previously related to cell division and regulatory volume decrease (RVD) [27–29]. Therefore, the expression of ANO1 in our system could be related to compensation of osmotic homeostasis after stress produced by the tissue disruption process needed to generate ICC primary cultures.

In prostate cancer, evidence suggests that ANO1 could regulate swelling-activated $Ca^{2+}$ entry through BCL2 activation and could thus regulate calcium homeostasis in these cells [28]. ANO1 has also been found overexpressed in gastrointestinal stromal tumors [30, 31], which originate from the ICC and present high BCL2 levels. However, the role of ANO1 expression regulation over BCL2 and over calcium oscillations remains to be investigated.

ANO1 $Ca^{2+}$-activated $Cl^-$ channels play an important role in slow wave generation. A recent study with *Tmem16a*$^{-/-}$ mice showed the complete loss of pacemaker activity in the muscle of the *Tmem16a*$^{-/-}$ mouse antrum and small intestine, whereas the ICC network and *c-Kit* immunoreactivity appeared normal [24]. Loss of pacemaker activity was found in both *W/W*$^{v}$ and *Tmem16a*$^{-/-}$ mice, suggesting that ANO1 shares the same functional significance as traditional ICC marker *c-Kit* at the mRNA level. Our study showed the higher success of single-cell PCR from ICC using *Ano1*, so *Ano1* is a better marker than *c-Kit* for transcript analysis of single ICC. We expect that the use of *Ano1* as a marker for single cell identification at the mRNA level will increase the success rate of further single ICC PCR experiments in the field, allowing validation and faster up-scaling to medium and high-throughput platforms.

**Acknowledgements.** This study was supported by a Natural Sciences and Engineering Research Council (NSERC) research grant to Jan D. Huzinga (#386877). Raúl Loera-Valencia was supported by CONACYT (Consejo Nacional de Ciencia y Tecnología, México, scholarship 290618). George W.J. Wright was supported by both an Ontario Graduate Scholarship and an NSERC Postgraduate Scholarship. We are grateful to Dr. Waliul Khan for the use of his thermal cycler and documentation system.

## REFERENCES

1. Huizinga, J.D., Thuneberg, L., Kluppel, M., Malysz, J., Mikkelsen, H.B. and Bernstein, A. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. **Nature** 373 (1995) 347–349. DOI: 10.1038/373347a0.
2. Koh, S.D., Sanders, K.M. and Ward, S.M. Spontaneous electrical rhythmicity in cultured interstitial cells of cajal from the murine small intestine. **J. Physiol.** 513 (Pt 1) (1998) 203–213.
3. Thomsen, L., Robinson, T.L., Lee, J.C., Farraway, L.A., Hughes, M.J., Andrews, D.W. and Huizinga, J.D. Interstitial cells of Cajal generate a rhythmic pacemaker current. **Nat. Med.** 4 (1998) 848–851.
4. Koh, S.D., Ward, S.M., Ordog, T., Sanders, K.M. and Horowitz, B. Conductances responsible for slow wave generation and propagation in interstitial cells of Cajal. **Curr. Opin. Pharmacol.** 3 (2003) 579–582.
5. Sanders, K.M., Koh, S.D. and Ward, S.M. Interstitial cells of cajal as pacemakers in the gastrointestinal tract. **Annu. Rev. Physiol.** 68 (2006) 307–343. DOI: 10.1146/annurev.physiol.68.040504.094718.
6. Huizinga, J.D., Berezin, I., Chorneyko, K., Thuneberg, L., Sircar, K., Hewlett, B.R. and Riddell, R.H. Interstitial cells of Cajal: pacemaker cells? **Am. J. Pathol.** 153 (1998) 2008–2011.
7. Huizinga, J.D. Gastrointestinal peristalsis: joint action of enteric nerves, smooth muscle, and interstitial cells of Cajal. **Microsc. Res. Tech.** 47 (1999) 239–247. DOI: 10.1002/(SICI)1097-0029(19991115)47:4 < 239::AID-JEMT3 > 3.0.CO;2-0.
8. Huizinga, J.D., Robinson, T.L. and Thomsen, L. The search for the origin of rhythmicity in intestinal contraction; from tissue to single cells. **Neurogastroenterol. Motil.** 12 (2000) 3–9.
9. Chen, H., Ordog, T., Chen, J., Young, D.L., Bardsley, M.R., Redelman, D., Ward, S.M. and Sanders, K.M. Differential gene expression in functional

classes of interstitial cells of Cajal in murine small intestine. **Physiol. Genomics** 31 (2007) 492–509. DOI: 10.1152/physiolgenomics.00113.2007.

10. Zhu, M.H., Kim, T.W., Ro, S., Yan, W., Ward, S.M., Koh, S.D. and Sanders, K.M. A Ca(2+)-activated Cl(-) conductance in interstitial cells of Cajal linked to slow wave currents and pacemaker activity. **J. Physiol.** 587 (2009) 4905–4918. DOI: 10.1113/jphysiol.2009.176206.
11. Sanders, K.M., Zhu, M.H., Britton, F., Koh, S.D. and Ward, S.M. Anoctamins and gastrointestinal smooth muscle excitability. **Exp. Physiol.** 97 (2012) 200–206. DOI: 10.1113/expphysiol.2011.058248.
12. Zhu, M.H., Sung, I.K., Zheng, H., Sung, T.S., Britton, F.C., O'Driscoll, K., Koh, S.D. and Sanders, K.M. Muscarinic activation of Ca2+-activated Cl-current in interstitial cells of Cajal. **J. Physiol.** 589 (2011) 4565–4582. DOI: 10.1113/jphysiol.2011.211094.
13. Eberwine, J. Single-cell molecular biology. **Nat. Neurosci.** 4 Suppl (2001) 1155–1156. DOI: 10.1038/nn1101-1155.
14. Takeda, Y., Koh, S.D., Sanders, K.M. and Ward, S.M. Differential expression of ionic conductances in interstitial cells of Cajal in the murine gastric antrum. **J. Physiol.** 586 (2008) 859–873. DOI: 10.1113/ jphysiol.2007.140293.
15. Wouters, M.M., Gibbons, S.J., Roeder, J.L., Distad, M., Ou, Y., Strege, P.R., Szurszewski, J.H. and Farrugia, G. Exogenous serotonin regulates proliferation of interstitial cells of Cajal in mouse jejunum through 5-HT2B receptors. **Gastroenterology** 133 (2007) 897–906. DOI: 10.1053/ j.gastro.2007.06.017.
16. Li, H.H., Gyllensten, U.B., Cui, X.F., Saiki, R.K., Erlich, H.A. and Arnheim, N. Amplification and analysis of DNA sequences in single human sperm and diploid cells. **Nature** 335 (1988) 414–417. DOI: 10.1038/ 335414a0.
17. Wang, B., Kunze, W.A., Zhu, Y. and Huizinga, J.D. In situ recording from gut pacemaker cells. **Pflugers Arch.** 457 (2008) 243–251. DOI: 10.1007/ s00424-008-0513-6.
18. Parsons, S.P., Kunze, W.A. and Huizinga, J.D. Maxi-channels recorded in situ from ICC and pericytes associated with the mouse myenteric plexus. **Am. J. Physiol. Cell Physiol.** 302 (2012) C1055–1069. DOI: 10.1152/ ajpcell.00334.2011.
19. Dubois, C.M., Ruscetti, F.W., Stankova, J. and Keller, J.R. Transforming growth factor-beta regulates c-kit message stability and cell-surface protein expression in hematopoietic progenitors. **Blood** 83 (1994) 3138–3145.
20. Gomez-Pinilla, P.J., Gibbons, S.J., Bardsley, M.R., Lorincz, A., Pozo, M.J., Pasricha, P.J., Van de Rijn, M., West, R.B., Sarr, M.G., Kendrick, M.L., Cima, R.R., Dozois, E.J., Larson, D.W., Ordog, T. and Farrugia, G. Ano1 is a selective marker of interstitial cells of Cajal in the human and mouse gastrointestinal tract. **Am. J. Physiol. Gastrointest. Liver Physiol.** 296 (2009) G1370–1381. DOI: 10.1152/ajpgi.00074.2009.
21. Kashyap, P., Gomez-Pinilla, P.J., Pozo, M.J., Cima, R.R., Dozois, E.J., Larson, D.W., Ordog, T., Gibbons, S.J. and Farrugia, G. Immunoreactivity for

Ano1 detects depletion of Kit-positive interstitial cells of Cajal in patients with slow transit constipation. **Neurogastroenterol. Motil.** 23 (2011) 760–765. DOI: 10.1111/j.1365-2982.2011.01729.x.

22. Parsons, S.P. and Huizinga, J.D. Transient outward potassium current in ICC. **Am. J. Physiol. Gastrointest. Liver Physiol.** 298 (2010) G456–466. DOI: 10.1152/ajpgi.00340.2009.
23. Wright, G.W., Parsons, S.P. and Huizinga, J.D. $Ca^{2+}$ sensitivity of the maxi chloride channel in interstitial cells of Cajal. **Neurogastroenterol. Motil.** 24 (2012) e221–234. DOI: 10.1111/j.1365-2982.2012.01881.x.
24. Phillips, J.K. and Lipski, J. Single-cell RT-PCR as a tool to study gene expression in central and peripheral autonomic neurones. **Auton. Neurosci.** 86 (2000) 1–12. DOI: 10.1016/S1566-0702(00)00245-9.
25. Hwang, S.J., Blair, P.J., Britton, F.C., O'Driscoll, K.E., Hennig, G., Bayguinov, Y.R., Rock, J.R., Harfe, B.D., Sanders, K.M. and Ward, S.M. Expression of anoctamin 1/TMEM16A by interstitial cells of Cajal is fundamental for slow wave activity in gastrointestinal muscles. **J. Physiol.** 587 (2009) 4887–4904. DOI: 10.1113/jphysiol.2009.176198.
26. Espinosa-Luna, R., Collins, S.M., Montano, L.M. and Barajas-Lopez, C. Slow wave and spike action potentials recorded in cell cultures from the muscularis externa of the guinea pig small intestine. **Can. J. Physiol. Pharmacol.** 77 (1999) 598–605.
27. Okada, Y., Shimizu, T., Maeno, E., Tanabe, S., Wang, X. and Takahashi, N. Volume-sensitive chloride channels involved in apoptotic volume decrease and cell death. **J. Membr. Biol.** 209 (2006) 21–29. DOI: 10.1007/s00232-005-0836-6.
28. Shen, M.R., Yang, T.P. and Tang, M.J. A novel function of BCL-2 overexpression in regulatory volume decrease. Enhancing swelling-activated Ca(2+) entry and Cl(-) channel activity. **J. Biol. Chem.** 277 (2002) 15592–15599. DOI: 10.1074/jbc.M111043200.
29. Ponce, A., Jimenez-Pena, L. and Tejeda-Guzman, C. The role of swelling-activated chloride currents (I(CL,swell)) in the regulatory volume decrease response of freshly dissociated rat articular chondrocytes. **Cell Physiol. Biochem.** 30 (2012) 1254–1270. DOI: 10.1159/000343316.
30. West, R.B., Corless, C.L., Chen, X., Rubin, B.P., Subramanian, S., Montgomery, K., Zhu, S., Ball, C.A., Nielsen, T.O., Patel, R., Goldblum, J.R., Brown, P.O., Heinrich, M.C. and van de Rijn, M. The novel marker, DOG1, is expressed ubiquitously in gastrointestinal stromal tumors irrespective of KIT or PDGFRA mutation status. **Am. J. Pathol.** 165 (2004) 107–113. DOI: 10.1016/S0002-9440(10)63279-8.
31. Robinson T.L., Sircar, K., Hewlett B.R., Chorneyko K., Riddell R.H. and Huizinga J.D. Gastrointestinal stromal tumors may originate from a subset of CD34-positive interstitial cells of Cajal. **Am. J. Pathol.** 156 (2000) 1157–1163.