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Inherent pacemaker function of duodenal GIST

Shinji Furuzono[c], *Susumu Ohya*[c], *Soichiro Inoue*[b], *Akimasa Nakao*[b], *Yuji Imaizumi*[c], *Shinsuke Nakayama*[a,*]

[a]*Department of Cell Physiology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Aichi, Nagoya 466-8550, Japan*
[b]*Department of Surgery II, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan*
[c]*Department of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya 467-8603, Japan*

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ABSTRACT

Gastrointestinal stromal tumours (GIST) are thought to derive from interstitial cells of Cajal (ICCs), which are putative pacemaker cells for gut motility. Isolated cells were obtained by enzymatic treatment of human duodenum GIST tissue having a frequent gain-of-function gene mutation. After cell culturing, c-Kit immunoreactivity was preserved and the cells developed long processes. Whole cell patch clamp recordings revealed voltage-dependent outward currents, without transient inward currents. Intracellular Ca^{2+} measurements showed oscillation-like spontaneous activity in some GIST cells. RT-PCR revealed expression of ion channels (Kv1.1, Kv1.6 and KCNH2; IP3R1, and IP3R2; TRPC1, 3, 6 and 7; Cx43), which have been suggested to play important roles in pacemaker activity. However, SCN5A, a TTX-resistant Na^+ channel known to be expressed in human ICCs, was below detectable levels. These data suggest that GIST cells appear to preserve some, but not all ionic mechanisms underlying pacemaker activity in ICC.

1. Introduction

There are small populations of cells which play important roles in numerous systems, and tumours of these cells would provide excellent opportunities to investigate the mechanisms underlying their function. Gastrointestinal stromal tumours (GIST), the most common mesenchymal tumours of the human gastrointestinal tract are thought to derive from interstitial cells of Cajal (ICC) [1], the putative pacemakers for gastrointestinal motility. A network of these cells is found in the myenteric plexus.

GIST are induced by mutation of the receptor tyrosine kinase, c-Kit [2,3]. In mice and guinea-pigs, c-Kit-immunoreactivity studies have identified the pacemaker activity of ICC [4,5]. However, only 'supporting' evidence has been presented for human ICC [6,7]. For example, in patients with diabetes mellitus, it has been reported that the number of ICC tends to decrease in the stomach, which might account for impaired gastric motility. Furthermore, in humans, the contribution of TTX-resistant Na^+ channels, in addition to voltage-operated Ca^{2+} channels, has been suggested for ICC pacemaking activity [8]. For clinical treatment of gastrointestinal dismotility

* *Corresponding author*: Tel.: +81 52 741 2111/744 2045; fax: +81 52 744 2048.
E-mail address: h44673a@nucc.cc.nagoya-u.ac.jp (S. Nakayama).
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including human, it is important to elucidate and/or confirm the mechanisms underlying gastrointestinal pacemaker activity. In the present study, using a duodenal GIST specimen, we have examined possible involvement of several mechanisms that have been proposed for ICC pacemaker activity.

2. Materials and methods

2.1. Cultured GIST cells

A duodenal specimen was obtained, under informed consent, from a 47-year-old male patient with duodenal GIST and synchronous multiple hepatic metastases. The GIST specimen was cut into small strips (1–2 mm in width, 10 mm in length). The strips were incubated overnight at 4 °C in a Ca^{2+}/Mg^{2+}-free solution containing digestive enzymes [collagenase (1–2 mg/ml; Wako Chemical, Osaka, Japan), disperse (500 PU/ml; Yakult, Tokyo, Japan); bovine albumin (16 mg/ml; Sigma, St. Louis, MO, USA)]; and then triturated with glass pipettes into an enzyme-free solution. The resultant isolated GIST cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% foetal bovine serum (Sigma) and antibiotics (streptomycin 30 μg/ml and penicillin 30 IU/ml; Sigma) at 37 °C for up to 3 weeks. Changes in cell shape were observed until day 16 of culture. In some preparations, expression of KIT was checked by staining with phycoerythrin (PE)-conjugated anti-human CD117 antibody (YB5.B8; eBioscience, San Diego, CA, USA) in 1/100–1/200 v/v.

2.2. Whole cell patch clamp and $[Ca^{2+}]_i$ measurements

Membrane current recordings and measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) were carried out in cultured GIST cells using essentially the same methods as previously described for cultured cell cluster preparations [9,10]. Briefly, whole cell membrane currents were measured at room temperature in voltage-clamp mode using a patch clamp amplifier (Axopatch 200A: Axon Instruments, Foster City, CA, USA), and digitised through an AD/DA converter (TL-1; Axon Instruments). A cut-off frequency of 2 kHz was applied to reduce noise. The resistance of the patch pipette was 2.5–5 MΩ, when a K^+-rich pipette solution was used. For $[Ca^{2+}]_i$ measurements, the cultured GIST cells were incubated for 3–4 h in 'normal' solution containing 8 μM fluo-3/AM and detergents (0.02% Pluronic F-127, Dojindo; 0.02% cremophor EL; Sigma). A CCD camera system (Argus HiS-CA; Hamamatsu Photonics, Hamamatsu, Japan) combined with an inverted microscope (Axiovert S100TV; Zeiss, Germany) was used to continuously monitor digital images of fluo-3 emission light (excitation at 488 nm; emission light of 515–565 nm). GIST cells were maintained at 35 °C on a micro-warm plate (MP10DM; Kitazato Supply, Fujinomiya, Japan).

The composition of 'normal' bathing solution was as follows (mM): NaCl, 125; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; glucose, 11.8; Hepes, 11.8; and pH adjusted to 7.4 with Tris base. The Ca^{2+}/Mg^{2+}-free solution, used for cell isolation, was made by iso-osmotic substitution with NaCl. The composition of the pipette solution was (mM): K-aspartate, 110; KCl, 20; $MgCl_2$, 4; EGTA (ethyleneglycol-bis-(β-aminoethylether) *N*,*N*,*N*′,*N*′-tetraacetic acid), 0.1; ATP, 4; GTP, 0.1; Hepes 20 (pH 7.2). Chemicals used were ATP (disodium salt) and GTP (trisodium salt) from Seikagakukogyo (Tokyo, Japan) and EGTA (free acid) from Sigma (St. Louis, MO, USA).

2.3. RT-PCR and sequencing

Total RNA was extracted from patient GIST, as previously reported [11]. ™II RNase H^- (Invitrogen, Carlsbad, CA, USA) and 200 μg/ml of random hexamer were used to reverse transcribe the RNA sample.

Real-time quantitative PCR was performed using Syber Green chemistry on an ABI 7000 sequence detection system (PE Biosystems). For PCR primers, see on-line Supplemental Table. Standard curves were drawn by regression analysis of the mean values for multiplex RT-PCR products of the $\log_{10}$-diluted cDNA. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an endogenous standard. All experiments were performed in triplicate. The specificity of each PCR product (amplicon in each band) was confirmed by sequencing with the chain termination method (ABI PRIZM model 3100) (PE Biosystems, Hayward, CA, USA).

In separate examinations to determine the mutation site, PCR products for exon 11 of c-Kit were cloned and sequenced as previously described by [12]. The primers used were as follows: forward: 5′-CCA GAG TGC TCT AAT GAC TG-3′; reverse: 5′-ACT CAG CCT GTT TCT GGG AAA CTC-3′.

2.4. Statistics

Numerical data are expressed as means ± standard deviation (SD).

3. Results

Isolated cells were prepared by triturating duodenal GIST tissue after treating with digestive enzymes, and then kept in culture medium for 3 weeks. RNA was also extracted from the duodenum GIST, and cDNA was reverse transcribed. Sequencing of the PCR products revealed that the c-Kit proto-oncogene underwent in-frame deletion of 6 bp in the juxtamembrane (JM) domain of exon 11 (Table 1), which is known to be the most frequent gain-of-function mutation [2,12,13].

Fig. 1A shows changes in the shape of isolated GIST cells during culture. On day 1 after isolation only round cells were observed (Fig. 1A(a)), although thin short processes were observed on the cell surface just after isolation (not shown). The diameter was 20–40 μm (26.7 ± 6.9 μm, $n = 50$). After 1 week, isolated GIST cells became oval-shaped with longer and shorter axes with some having processes as long as the major axis of the cells (Fig. 1A(b) and (c)). After further culturing, GIST cells developed long and multiple processes (Fig. 1A(d)–(f)), and fusion of the processes between neighbouring GIST cells (Fig. 1A(e)) like the ICC network in the myenteric plexus. Most GIST cells showed c-Kit-immunoreactivity (Fig. 1B) even after day 12.

Whole cell membrane currents were recorded from isolated GIST cells cultured for 8–16 days, using a K^+-rich solution in the pipette. The capacitance of the GIST cell membrane was 30.4 ± 4.1 pF ($n = 6$). Fig. 2A shows an example of membrane currents elicited by rectangular voltage steps of

Table 1 – Gain-of-function deletion mutation in GIST sample

	550 ▼	560 ▼	570 ▼	580 ▼	590 ▼
WT	KPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLS				
GIST sample	KPMYEVQ	VVEEINGNNYVYIDPTQLPYDHKWEFPRNRLS			

The amino acid sequence is compared to the wild type (WT).

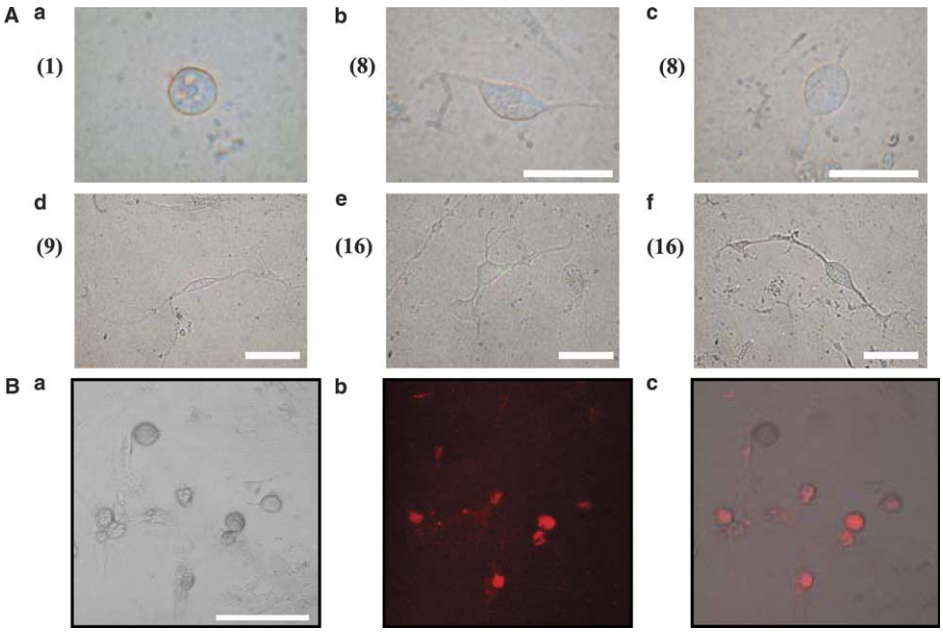

Fig. 1 – Isolated GIST cells maintained in a culture medium. (A) Panels (a)–(f) show changes in the shape of GIST cells after 1–16 days of culture. Scale bars = 50 μm. Note growth of processes. (B) c-Kit-immunoreactivity of GIST cells: (a) transmission image; (b) fluorescent image with PE-conjugated c-Kit antibody (YB5.B8) and (c) merged image of (a) and (b).

−70 to +130 mV. At positive test potentials, the outward currents clearly developed in a time-dependent manner. As the positivity of the test step increased, the amplitude increased and the rising time constant decreased progressively. Inward current was not evoked at any voltage step. The properties of membrane currents suggest the existence of voltage-dependent K^+ channels.

In mouse small intestine preparations, we previously suggested that $[Ca^{2+}]_i$ oscillation is the primary mechanism for pacemaking in ICC, and thereby, Ca^{2+}-activated ion channels

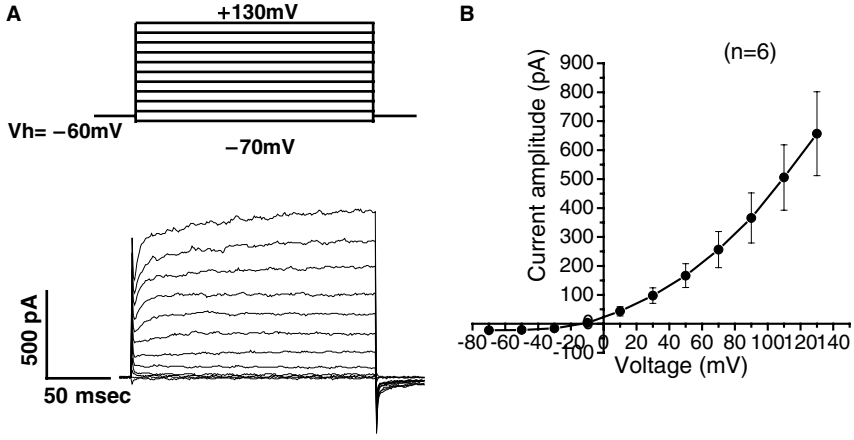

Fig. 2 – Whole cell membrane current recorded from isolated GIST cells cultured after 8–16 days. (A) Example of current traces obtained by applying a set of rectangular pulses. The holding potential was −60 mV. (B) Current–voltage relationship. The peak amplitude of the membrane current was plotted against the voltage of the rectangular pulse applied.

in the plasmalemma (e.g. Ca^{2+}-activated Cl^- channels) are periodically activated [14,15]. Therefore, we measured the fluorescence of a Ca^{2+}-sensitive dye fluo-3 in GIST cells. Fig. 3 shows oscillation-like spontaneous $[Ca^{2+}]_i$ activities recorded in some GIST cells on day 16 of culture. The majority of cells had no spontaneous Ca^{2+} activity. $[Ca^{2+}]_i$ activities were not synchronised, and the amplitude of $[Ca^{2+}]_i$ rise ($\Delta F/F_0$) was 0.8–1.4 (1.13 ± 0.33, $n = 4$). These observations suggest that GIST cells preserve the essential mechanism in pacemaking, although normal cell-to-cell coupling is not constituted.

To confirm the membrane currents and $[Ca^{2+}]_i$ activities in GIST, we examined the possibility of ion channel expression, using RT-PCR. In GIST tissue, the expression of c-Kit was approximately 0.6, relative to GAPDH. Among the three types of $InsP_3R$ (intracellular Ca^{2+} release channels), type 1 (IP3R1) was predominant. The relative expression to GAPDH was 0.024, 0.003, and 0.001 for IP3R1, IP3R2, and IP3R3, respectively (Fig. 4A). Canonical transient receptor potential (TRPC) channels are candidates of Ca^{2+} influx pathways essential to maintain $[Ca^{2+}]_i$ oscillation. Of the seven TRPC channels examined, TRPC1 was predominant (0.045 relative to GAPDH), and TRPC3, 6 and 7 were detectable (Fig. 4B). To detect molecular candidates for the voltage-dependent outward current shown in Fig. 3, we also examined the expression levels of voltage-dependent K^+ (Kv) channel subtypes: Kv1.1–1.7, Kv2.1–2.2, Kv3.1–3.4, Kv4.1–4.3, KCNH1–8 and KCNQ1–5. Of the 29 Kv channel subtypes examined, the signals of Kv1.1, 1.6, and KCNH2 (HERG) transcripts were detected in GIST (Fig. 4C), whereas the signals of the other Kv subtypes were very weak or undetectable (data not shown). The relative expression to GAPDH was 0.005, 0.004, and 0.008 for Kv1.1, 1.6 and KCNH2, respectively.

Gap junction channels support cell-to-cell coupling in GI spontaneous rhythmicity [16,17], and the existence of TTX-resistant Na^+ channels (SCN5A) has been shown in human ICC [8]. Of the three subtypes of connexin (Cx) examined, Cx43 was predominant. Expression relative to GAPDH was 0.005, 0.001 and 0.108 for Cx37, 40 and 43, respectively. The SCN5A signal was undetectable.

4. Discussion

GIST are thought to differentiate towards ICC (interstitial cells of Cajal), putative gastrointestinal pacemaker cells, because both GIST and ICC express common cell markers (CD117 and CD34) [12]. In the present study, we examined several biological properties of GIST cells. The GIST cells used had an in-frame deletion in the JM domain, which is known to be the most frequent type of gain-of-function mutation [13]. First, we observed that during culture, multiple processes developed from GIST cells, and that some of them had contact with those of neighboring cells, mimicking the pattern in ICC

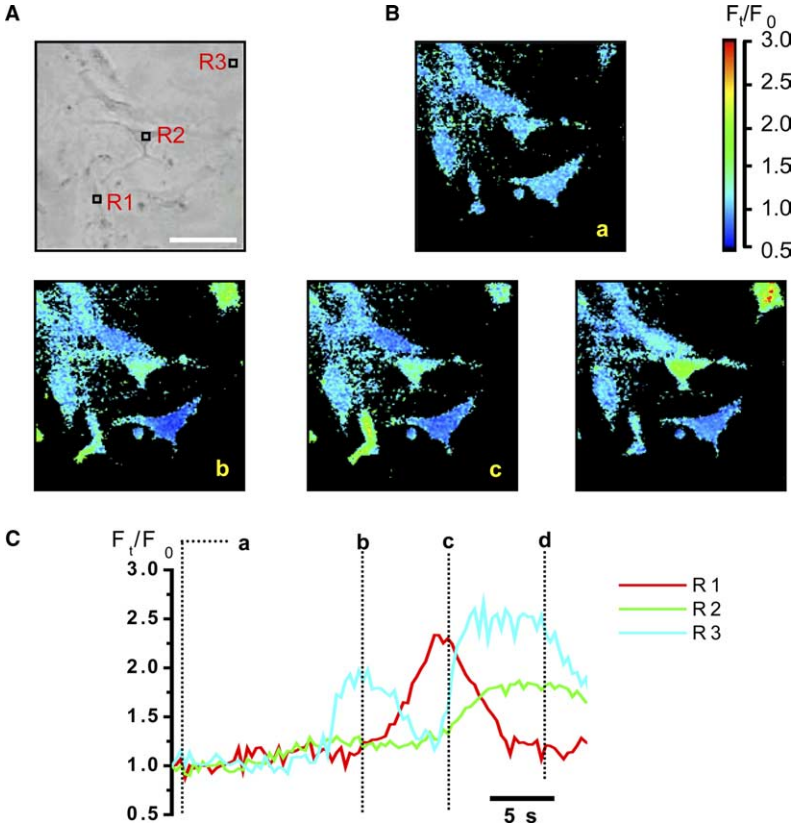

Fig. 3 – Spontaneous $[Ca^{2+}]_i$ activity in GIST cells. (A) Transmission image. (B) $[Ca^{2+}]_i$ images measured using fluo-3 (a)–(d). (C) Changes in $[Ca^{2+}]_i$ measured in the regions indicated in A (a). Fluo-3 fluorescence was used as an index of $[Ca^{2+}]_i$ activity. $[Ca^{2+}]_i$ images were normalised by the fluorescent intensity of the initial image (acquired at t = 0). The acquisition time of the $[Ca^{2+}]_i$ image is indicated in C.

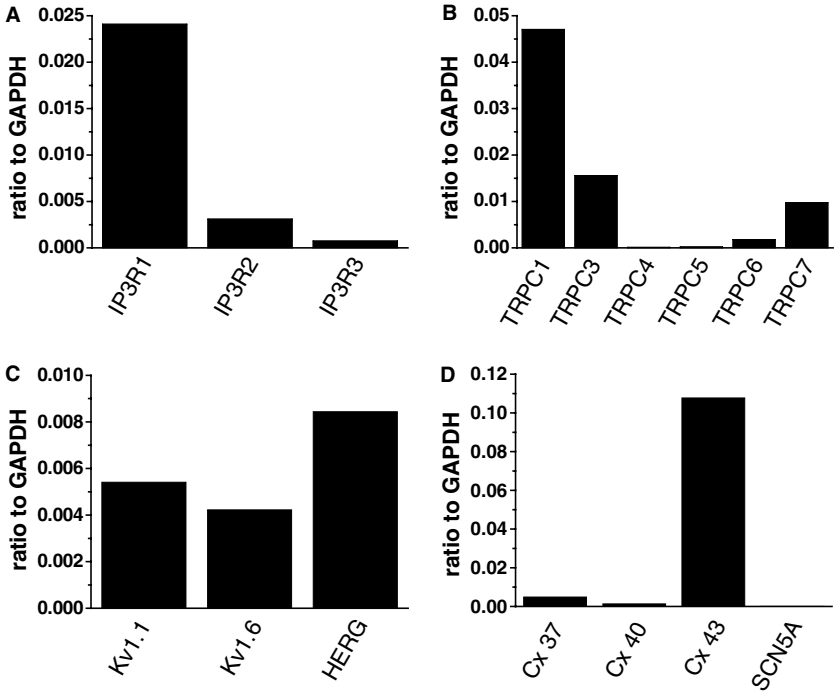

Fig. 4 – Quantitative RT-PCR examinations of GIST cells. Each cDNA expression is shown relative to GAPDH. Graphs show expressions of: (A) $InsP_3R$; (B) TRPC; (C) Kv channels; and (D) Cx and SCN5A.

which forms a network in the myenteric plexus. These morphological observations support the hypothesis that GIST cells may differentiate towards ICC.

It has been suggested that $[Ca^{2+}]_i$ oscillations play a crucial role in mouse ICC pacemaking [10,14,15,18]. Previous animal studies have suggested that intracellular Ca^{2+} release channels ($InsP_3R$ [15,19]) and Ca^{2+} influx pathways, other than L-type Ca^{2+} channels (TRP channels [10,15]) are important in the process of $[Ca^{2+}]_i$ oscillations. The present study provides evidence, consistent with previous animal studies, for the mechanisms underlying gastrointestinal pacemaker activity. Firstly, some GIST cells show oscillation-like $[Ca^{2+}]_i$ activity (Fig. 3). Secondly, RT-PCR examinations demonstrate the presence of the essential ion channel members for $[Ca^{2+}]_i$ oscillations: IP3R1 and 2; TRPC1, 3, 6 and 7. Thirdly, Kv family channels (Kv1.1, 1.6 and KCNH2) are expressed. The observed *I–V* relationship (progressive increase in the amplitude with increasing positivity of the step) and the relatively rapid activation and inactivation characteristics do not suggest a major contribution of KCNH2 [20], despite the highest expression of the three channels. Kv1.1 and 1.6 are presumably responsible for the outward currents recorded in the whole cell mode patch clamp experiments. These channels would play an important role in hyperpolarizing the slow pacemaker potentials that last for several seconds [21,22].

In human ICC, TTX-resistant Na^+ channels have been shown to play a role [8]. However, we did not observe voltage-dependent inward currents. In support of this fact, SCN5A is below detectable level in RT-PCR studies. Also, it is thought that gap junction channels play a role in synchronising pacemaker activities between ICCs [16,17]. RT-PCR demonstrated considerable expression of gap junction channels, especially Cx43, which has been shown to respond to Ras signalling [23,24]. Nevertheless, $[Ca^{2+}]_i$ oscillations are observed only occasionally, and are not synchronised between adjacent GIST cells. GIST cells appear to preserve some, but not all ionic mechanisms underlying pacemaker activity in ICC.

In the autonomic nervous system, there are many organs and tissues, other than heart and gastrointestinal tracts, which show spontaneous contractions. There is now an accumulating body of evidence that ICC-like pacemaker cells exist in these organs and tissues. [ureter [25]; urethra [26]; urinary bladder [27]; lymph ducts [28]]. Tumours of such cells might provide an excellent opportunity to study their original function.

It is speculated that chemical therapy, targeting the protein tyrosine kinase c-Kit, affects numerous functions in the autonomic nervous system through impairing ICCs and ICC-like pacemaker cells. Also, drugs affecting ion channels involved in pacemaker function would cause a large variety of modulation in spontaneous rhythmicity within numerous systems, if polymorphic changes exist [29]. These issues regarding pharmaceutical treatments, therefore, merit further investigation. In summary, the present study provides evidence that GIST cells, with a typical mutation of c-Kit, preserve several, but not all, important factors and mechanisms related to pacemaker function, and reinforcing the notion that GIST is a tumour derived from gastrointestinal pacemaker cells.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2005.09.024.

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