Hydrogen Sulfide, a Gaseous Transmitter, Stimulates Proliferation of Interstitial Cells of Cajal via Phosphorylation of AKT Protein Kinase

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Interstitial cells of Cajal (ICC) are distributed throughout the gastrointestinal (GI) tract and have important functions in the control of GI motility. Loss of ICC is associated with several GI motility disorders; yet, the mechanisms modulating ICC survival and proliferation are not fully understood. Hydrogen sulfide (H\textsubscript{2}S) has been reported to be a gaseous transmitter that regulates cellular proliferation. This study aims to establish whether H\textsubscript{2}S participates in regulation of ICC proliferation. The effect of H\textsubscript{2}S was studied in primary cultures of ICC, prepared from the mouse small intestine. To determine the extent of ICC proliferation, we used immunofluorescent staining to study alterations in the number of cells expressing c-Kit\textsuperscript* and CD44\textsuperscript*, markers for mature ICC. Phosphorylation of Akt was measured by Western blot analysis. Treatment with low concentrations of NaHS (H\textsubscript{2}S donor, 1-30 \(\mu\)M) showed no apparent toxicity, as judged from cell numbers. Importantly, treatment with NaHS (15 \(\mu\)M) for 24 hours increased the numbers of c-Kit\textsuperscript*/CD44\textsuperscript* ICC by 23.3 \(\pm\) 1.4\% \((P < 0.05)\). Moreover, NaHS increased Akt phosphorylation, which was prevented with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (5 \(\mu\)M). LY294002 also blocked the NaHS-mediated increase in the number of ICC. In addition, H\textsubscript{2}S enhanced the proliferation of mature ICC in the \textit{in vitro} culture system used here in a concentration-dependent manner. The present study suggests that H\textsubscript{2}S may be a critical factor in maintaining ICC numbers and may have a novel, Akt-dependent role in proliferation of mature ICC.

**Keywords:** interstitial cell of Cajal; hydrogen sulfide; immunofluorescence technique; cell culture; Akt signaling pathway

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capacities of compensating mechanisms, including signaling via other receptor tyrosine kinases and factors, such as NO (Choi et al. 2007) and CO (Choi et al. 2008), as mouse strains differ in their sensitivity to in vivo c-Kit blockade (Maeda et al. 1992; Torihashi et al. 1999). These apparent redundancies may, in fact, be cytoprotective for ICC. Accumulating evidence has shown that H2S effectively modulates cell proliferation in a variety of different systems (Deplancke and Gaskins 2003; Cai et al. 2007). Whether or not H2S also stimulates the proliferation of ICC as does NO and CO is a very important area of exploration.

Morphologists have noted the close relationship between ICC and nerve fibers for many years. A study on guinea pig and rat colons has shown the close association between nerve fibers and ICC (Komuro 1999). Electron microscopy has revealed that enteric motor nerve terminals form close anatomical contacts (< 10 nm) with ICC-IM (intramuscular ICC) (Beckett et al. 2005). Hence, ICC injury is usually associated with injury to the enteric nervous system (Huizinga and White 2008). Schicho et al. (2006) have demonstrated that more than 90% of guinea pig and human submucous and myenteric neurons coexpress CSE and CBS. In that study, myenteric ICC were CSE-immunoreactive, demonstrating that ICC may produce H2S that acts in an autocrine manner, again suggesting a close correlation between H2S and ICC.

CD44 mRNA is expressed in isolated small intestinal ICC. No other major cell type in the small intestine appears to express CD44 (Chen et al. 2007). Lorincz et al. (2008) found that mature ICC are Kit+CD44+CD34+Insr Igf1r+ and that all ICC express CD44, which is expressed throughout ICC differentiation. Thus, CD44 may be utilized as a surrogate ICC marker. Utilizing a double-labeling method with the markers c-Kit and CD44 to observe the changes in the numbers of mature ICC, the present study focuses on the role of H2S in mature ICC proliferation in a series of in vitro experiments. Additionally, the intracellular signaling pathways involved in the proliferative role of H2S are examined.

Materials and Methods

Animals

BALB/c mice of either sex between 2 and 4 days old were purchased from the Animal Research Center of Daping Hospital, affiliated with Third Military Medical University (Chongqing, China). Mice were bred and maintained in specific pathogen-free facilities, and ethical approval and guidelines were provided by the Institutional Animal Care and Use Committee at Third Military Medical University and followed for all animal experimentation. They were killed by CO2 inhalation and decapitation. The GI tract, starting from the esophagus to the colon, was removed with an intact mesenteric vascular bed to minimize stretching on transfer to a dissection dish, which was filled with M199 medium (Gibco, Carlsbad, California, USA). After the gut was removed from the mesenteric vascular bed under a dissection microscope, the small intestine (1.5 cm length) was obtained and mounted without stretching onto a Sylgard surface with insect pins (0.1 mm in diameter). For all experiments, the mucosa and mesentery were removed using a binocular microscope.

Chemicals

H2S was administered in the form of NaHS (Sigma, St. Louis, Missouri, USA), which has been well established as a reliable donor of H2S (Abe and Kimura 1996; Zhao et al. 2001; Deplancke and Gaskins 2003; Li et al. 2005). When NaHS is dissolved in saline, about one-third of H2S exists as undissociated gas, and the remaining two-thirds is HS−. The concentrations of NaHS for the present study did not affect the pH values of the culture medium. NaHS stock solution was freshly prepared on the day of the experiment. Dulbecco’s modified Eagle’s medium (DMEM), sodium pyruvate, fetal bovine serum (FBS), and the antibiotic-antimycotic were from Invitrogen (Carlsbad, California, USA). Of note, at 37°C, the concentration of H2S in the solution is very stable below 1 mM (Hosoki et al. 1997; Zhao and Wang 2002; Du et al. 2004). LY294002 was purchased from Calbiochem (San Diego, California, USA), and antibodies against ERK, p38, and Akt were obtained from Cell Signaling Technology (Boston, Massachusetts, USA).

Since the concentration of freshly prepared NaHS was far below 1 mM, we allowed the concentration of H2S to stabilize in the culture solution for 24 hours. Primary cell cultures were treated daily with or without NaHS. The NaHS solution was filter-sterilized immediately before its addition. To minimize the loss and spontaneous oxidation of volatile, redox-labile HS−, vented caps were immediately replaced with unvented caps for the entire length of the experimental incubation. Importantly, various concentrations of H2S used in the proliferative aspects of this study are on the lower end of the general range of those found physiologically (as discussed above).

Enzymatic dissociation of ICC

Freshly dispersed cells were obtained from murine small intestinal muscle strips. The small intestine was removed and pinned down in a Sylgard-lined dish containing Hank’s calcium-free buffer and 1% antibiotic-antimycotic. The muscle layers were peeled away and dissociated in a cocktail (pH 7.0) containing 2,500 U collagenase, 20 mg bovine serum albumin, 20 mg trypsin inhibitor, 5.5 mg adenosine triphosphate (all from Boster, Wuhan, China), and 10 mL Hank’s balanced salt solution at 32°C for 15 minutes in a gently shaken water bath. The tissue was then triturated and spun down at 2,100 g for 10 minutes. Cells were resuspended in 2 mL DMEM with 10% FBS, 1% sodium pyruvate, and 1% antibiotic-antimycotic.

Primary cultures of ICC

Based on the method developed by Wouters et al. (2007), freshly dispersed cells from the murine small intestine were cultured on 22-mm glass cover slips covered with 1/3/fourth mSCF248 murine stem cell factor-secreting fibroblasts (courtesy of Dr. Fan-ming Li, Fudan University, Shanghai, China) at 4.5 × 10⁴ cells per cover slip in high-glucose DMEM containing 10% fetal bovine serum, 2% antibiotic-antimycotic, and 1% sodium pyruvate. After 30 hours, fibroblast cell division was arrested by irradiation at 16,000 Gy. After a 24-hour recovery period, 250-µL suspensions containing cells that were freshly dissociated from mouse jejunum were plated onto the 1/3/fourth mSCF248 fibroblasts at a cell density of approximately 3×10⁴ cells per cover slip. Cells were allowed to incubate for 30 minutes at 37°C/5% CO2 before 2 mL of culture medium was added to the well. These culture conditions resulted in cell cultures that were highly enriched with ICC.
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Cell viability assay

After 2 days in culture, according to the method developed by Jin (Jin et al. 2006), with modifications, ICC at 1 × 10⁵ cells/ml were seeded in each well of a 96-well plate (n = 6) prior to the treatment with NaHS. After 24 hours with or without different concentrations (1-200 µM) of NaHS, 20 µL of a 5 mg/mL solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) tetrazolium substrate (Sigma, St. Louis, Missouri, USA) in phosphate-buffered saline (PBS) was added and incubated for 4 hours at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 150 µL of a 50% N,N-dimethyl formamide/10% SDS solution and incubated for 4 hours at room temperature. Sample absorbances were then measured on a microplate reader (Tecan, Männedorf, Switzerland) at 490 nm.

Immunohistochemistry

ICC in primary cell culture were identified by immunofluorescence double-labeling of c-Kit and CD44, as previously described (Rich et al. 2003). The culture medium was removed from the cover slips, and thereafter, all of the cells that were cultured with or without different concentrations of NaHS were fixed with acetone (4°C, 10 min), washed with PBS, incubated with 10% normal donkey serum (NDS), and 0.2% Triton-X-100 (Sigma, St. Louis, Missouri, USA) in 0.1 M PBS for 1 hour to minimize non-specific antibody binding, and incubated with the rat monoclonal anti-c-Kit antibody A2 (1:100) (eBioscience, San Diego, California, USA) diluted in 5% NDS for 2 hours at 37°C. Next, the sections were rinsed in PBS and incubated in the dark for 1 hour at room temperature with Cy3-conjugated secondary antibody (anti-rat IgG, 1:100) (Zymed, San Diego, California, USA), washed in PBS three times for 5 minutes, and incubated with FITC-conjugated rabbit polyclonal anti-CD44 antibody (1:100) (Zymed, San Diego, California, USA). The cells were washed in PBS three times for 5 minutes, and 4’, 6-diamidino-2-phenylindole (DAPI) (1/3000) (Invitrogen, Carlsbad, California, USA) was applied for 5 minutes as a nuclear counterstain. Cover slips were mounted with 30% glycerol (Sigma, St. Louis, Missouri, USA) and washed in PBS three times for 5 minutes again before being examined by confocal microscopy.

Analysis of ICC proliferation

After 24 hours with or without different concentrations of NaHS treatment, immunostained cultures were examined for positive cells with a TCS SP5 confocal laser scanning microscope (Leica, Bensheim, Germany) at appropriate excitation wavelengths for Cy3 (552 nm) and FITC (488 nm). A 100x objective was used to count the number of c-Kit/CD44 immunofluorescently double-labeled ICC-like cells per high-power field. One high-power field covered 0.038 mm². At least 35 high-power fields were counted per culture, since this is the optimal number in order to minimize field-to-field variations and avoid overcounting fields (Wouters et al. 2007).

Western blotting analysis

The cells were treated either with 15 µM NaHS for different durations (0-120 minutes) or with increasing doses of NaHS (1-30 µM) for 30 minutes. In another set of experiments, cells were treated for 30 minutes with LY 294002 (5 µM) or vehicle (M199), followed by stimulation with NaHS at 15 µM for 30 minutes. Cells were then lysed with 1 × SDS sample buffer (1% Triton X-100; 0.1% SDS; 150 mM NaCl; 50 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 7.5; 1% sodium deoxycholate; 1% Cocktail; 1 mM Na₃VO₃; 0.5 mM PMSF). Protein concentration was determined with the BCA reagent. Thirty micromegrams of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with TBST containing 5% milk for 1 hour, the membrane was incubated with antibodies against ERK, p38, or Akt overnight at 4°C. After incubation in horseradish peroxidase-conjugated secondary antibody for 1 hour, bands were scanned and quantified against the local background using the Bio-Rad Gel Doc system (Hercules, California, USA).

Measurement of NO production

Cells were stimulated with different concentrations of NaHS for 24 hours and then the supernatant was collected. The generation of NO was determined by measuring the stable NO metabolites (i.e., total nitrates) in the culture medium with a nitrite detection kit (Beyotime Biotech Inc, Jiangsu, China) as described elsewhere (Cai et al. 2007). Briefly, the medium (100 µl) was mixed with 100 µl of Griess reagent in a 96-well plate. Nitrite concentration was determined by spectrophotometry (540 nm) from a standard curve (0-100 µM), derived from NaNO2.

Statistical analysis

Data were expressed as means ± SE. Differences between groups were analyzed by one-way ANOVA, followed by post hoc test where applicable. Significance was established as P < 0.05.

Results

Low concentrations of NaHS show no toxicity in primary cultures of ICC

ICC were treated with or without different concentrations of NaHS (1-200 µM) for 24 hours. Treatment with relatively high concentrations of NaHS (60, 100, and 200 µM) showed apparent toxicity in primary cultures of ICC, as demonstrated by a significant reduction in cell viability.

![Fig. 1. The cell toxicity of H₂S on ICC. Cell viability was assessed using the MTT method, and ICC were treated with or without different concentrations of NaHS (1-200 µM) for 24 hours. Treatment with relatively high concentrations of NaHS (60, 100, and 200 µM) showed apparent toxicity in primary cultures of ICC and induced significant reduction in cell viability (n = 6, *P < 0.05). Treatment with low concentrations of NaHS (H₂S donor, 1-30 µM) showed no apparent toxicity (n = 6, P > 0.05).](image-url)
of 6.8 ± 1.5%, 19.2 ± 2.9%, and 30.2 ± 2.6% (n = 6, P < 0.05) below the percentage of cell viability of the control (100 ± 0.89%), respectively (Fig. 1). Moreover, with rising concentrations in NaHS (at relatively high concentrations), the viability of cultured cells showed a downward trend. The viability of the ICC at concentrations less than 60 µM was good, and there was no difference in viability among the groups (n = 6). Furthermore, treatment with low concentrations of NaHS (H₂S donor, 1-30 µM) showed no apparent toxicity. The concentrations of NaHS that we used in the following experiment were 1, 15, and 30 µM.

Number of ICC in primary cultures increases at low concentrations of NaHS

After 2 days in culture, these cells further developed into cells with prolonged processes, branches, and features that were similar to those of mature ICC. NaHS exogenously applied for 24 hours induced a dose-dependent saturable increase in c-Kit⁺/CD44⁺ ICC numbers in primary cultures. At 1 µM NaHS, no change was observed in ICC number compared to control (without NaHS) (3.34 ± 0.91 per field vs 3.20 ± 1.03, n = 6, P > 0.05) (Fig. 2A). An increase in numbers was first detected at 15 µM NaHS (3.95 ± 1.19 per field versus 3.20 ± 1.03, n = 6, P < 0.05) (Fig. 2B). An increase was also detected at 30 µM NaHS (3.90 ± 0.71 per field versus 3.20 ± 1.03, n = 6, P < 0.05) (Fig. 2C). This represents an increase of 23.3 ± 1.4% and 21.8 ± 2.2%, respectively. The proliferative effect was observed at 15 µM and 30 µM NaHS (Fig. 3). The effect of the former was better; therefore the concentration of NaHS that we used in the time-dependent experiment was 15 µM. These data suggest that low concentrations of NaHS treatment exert a direct growth-stimulating effect on mature ICC.

NaHS increases Akt phosphorylation

The PI3K downstream effector, Akt, was examined by Western blot analysis of cell extracts of ICC upon H₂S stimulation. ICC were treated with or without increasing concentrations of NaHS (1-60 µM) for 30 minutes. Akt phosphorylation was significantly increased by 92.2 ± 7.6% and 87.9 ± 6.7% following administration of NaHS at 15 and 30 µM, respectively (Fig. 4A). A single dose of NaHS (15 µM) induced a time-dependent increase in Akt phosphorylation,
Fig. 4. H₂S increases Akt phosphorylation without inducing phosphorylation of ERK or p38 in ICC. A, Effects of 30-minute treatment with various concentrations of NaHS (1-60 µM) on Akt phosphorylation. B, Time course of Akt phosphorylation induced by NaHS (15 µM). C, NaHS-induced Akt phosphorylation prevented by LY 294002 (5 µM). D and E, NaHS (15 µM) did not induce phosphorylation of ERK1/2 (D) or p38 (E) within 1 hour after treatment. Data represent the mean ± SE of six independent experiments. *P < 0.05.
which began at 10 minutes and peaked at 30 minutes (Fig. 4B). NaHS-induced Akt phosphorylation was abolished by LY294002, the PI3K inhibitor, suggesting that PI3K is the upstream regulator of Akt upon H$_2$S stimulation (Fig. 4C). In contrast, NaHS treatment for 1 hour (15 $\mu$M) did not change the degree of phosphorylation of ERK (Fig. 4D) and p38 (Fig. 4E), indicating that NaHS does not increase ERK or p38 phosphorylation.

**LY294002 blocks the increase in the number of ICC by NaHS**

ICC were treated for 30 minutes with LY294002 (5 $\mu$M), followed by stimulation with NaHS at 15 $\mu$M for 24 hours; no change was observed in ICC numbers compared to control (2.97 ± 0.87 per field vs 3.19 ± 0.98, n = 6, P > 0.05).

**NaHS shows no effect on the levels of NO metabolites**

Stimulation of ICC with increasing concentrations of NaHS (1-30 $\mu$M) did not change the levels of NO metabolites, nitrites, in the culture medium. The average NO metabolites were 0.22 ± 0.04 (control), 0.21 ± 0.07 (1 $\mu$M), 0.23 ± 0.06 (15 $\mu$M), and 0.21 ± 0.08 (30 $\mu$M), respectively.

**Discussion**

This study shows that low concentrations of exogenously applied H$_2$S (in the form of NaHS) increase mature ICC numbers *in vitro*, that the effect of H$_2$S on mature ICC numbers is dependent on activation of Akt, and that the effect is mediated in part by increased proliferation of ICC. Hence, H$_2$S may be a critical factor that regulates ICC numbers.

This effect of H$_2$S on ICC has not been previously reported. Indeed, the only well-accepted regulator of ICC survival and development is stem cell factor (Bernex et al. 1996; Ward et al. 1999; Rich et al. 2003), the ligand for the Kit tyrosine kinase receptor. Maeda et al. (1992) reported previously that blockade of Kit on alternate days from Day 0 to 8 postpartum (p.p.) by anti-Kit antibody induced a deficiency in ICC, but that an injection of anti-Kit antibody from Day 4 to 10 p.p. in neonates failed to induce a deficiency in ICC, suggesting that the SCF-Kit system is not absolutely required for the survival of the developed ICC. The presence of unrecognized factors, other than SCF, assists the survival of ICC under *in vivo* conditions. These may include NO (Choi et al. 2007), CO (Choi et al. 2008), 5-HT (Wouters et al. 2007), insulin or IGF (insulin-like growth factor)-I (Horvath et al. 2005), or IL-9 (Ye et al. 2006). In the present study, *in vitro* experiments strongly suggest that H$_2$S can directly affect ICC numbers. Furthermore, this study showed a proliferative effect of NaHS that was administered in ICC culture *in vitro* at lower concentrations of 15-30 $\mu$M.

Increased survival may be due to decreased cell death or increased proliferation, as both would result in increased numbers of ICC. Of note, c-Kit$^+$/CD44$^+$ cells in culture resembled mature ICC (Huizinga and White 2008). By a manual counting method, we confirmed that ICC appeared to increase with the addition of physiological concentrations of H$_2$S. When H$_2$S was given at concentrations less than 60 $\mu$M (in the form of NaHS) to cultured ICC, cell viability was not affected. In a dose-dependent manner, H$_2$S increased ICC numbers compared with control at physiologically relevant concentrations. At doses higher than 60 $\mu$M, H$_2$S was correlated with a significant reduction in cell viability, which may be related to its toxicity. At this time, the routine addition of NaHS to stimulate ICC proliferation cannot be recommended, because gaseous transmitters interact with each other *in vivo*. Furthermore, additional studies are required to evaluate the normal properties of ICC for longer culture periods.

The current study does establish that the novel proliferative effect of H$_2$S on ICC is dependent on the activation of the PI3K-AKT pathway *in vitro*, which results, at least in part, from an increase in the proliferation of ICC. The mechanisms by which H$_2$S promotes cell growth are not fully understood. In the rat intestinal crypt IEC-18 cell line, H$_2$S stimulates MAP kinase signaling pathways including ERK and p38, but not JNK, leading to up-regulation of c-Jun and enhanced proliferative activity (Deplancke and Gaskins 2003). In the present study, NaHS treatment induced a dose- and time-dependent increase in Akt phosphorylation in ICC. The PI3K inhibitor, LY 294002 prevented H$_2$S-induced Akt phosphorylation; moreover, it blocked the increase in the numbers of ICC by low concentrations of NaHS. Neither ERK nor p38 was activated by H$_2$S at a low concentration (in the form of 15 $\mu$M NaHS) in our study.

H$_2$S is a bifunctional modulator of cell survival, and it effectively modulates cell growth or death in different cells. Du et al. (2004) showed that NaHS at 100 and 500 $\mu$M suppressed the proliferation of rat aortic vascular smooth muscle cells (VSMCs) that was induced by endothelin-1 or fetal bovine serum, and Baskar et al. (2007) showed that H$_2$S at 10-75 $\mu$M caused a concentration-dependent increase in micronuclei formation (indicating DNA damage) and cell cycle arrest in G$_1$ phase in cultured human lung fibroblast. In contrast, H$_2$S increased endothelial cell proliferation at physiologically relevant concentrations (Deplancke and Gaskins 2003). It is particularly noteworthy that different concentrations of H$_2$S exerted different effects on the endothelial cell. The addition of 0.2-5 mM NaHS for 4 hours increased the proliferation of endothelial cells, whereas apoptosis occurred only at the highest concentration of H$_2$S (5 mM NaHS). Thus, H$_2$S could be toxic and also protective, depending on distinct experimental conditions, including the cell types being studied and its local concentration (Fiorucci et al. 2006).

H$_2$S is endogenously generated from cysteine metabolism; H$_2$S levels in the tissues and circulation have been reported to be 10-160 $\mu$M in several mammalian species, including mouse, rat, human, and bovine (Goodwin et al.
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1989; Abe and Kimura 1996; Zhao et al. 2001; Fiorucci et al. 2006; Yang and Wang 2007). As stated above, when NaHS was dissolved in saline, about one-third of H$_2$S existed as undissociated gas, and the remaining two-thirds was HS$^-$ (Abe and Kimura 1996; Zhao et al. 2001). Thus, with 15 $\mu$M NaHS employed in the present study, the concentration of H$_2$S was 5 $\mu$M, and was within the physiological levels.

With respect to interactions among gaseous transmitters, NO donors, for example, have previously been shown to increase the expression of CSE and the release of H$_2$S in cultured smooth muscle cells (Zhao et al. 2001). Conversely, H$_2$S inhibits NO generation and expression of inducible NO synthase (iNOS) in LPS-exposed macrophages through a mechanism that also appears to involve up-regulating heme oxygenase-1 (Oh et al. 2006). The effect of H$_2$S on the response to NO is more complicated. Certain studies (Hosoki et al. 1997) reported that low concentrations of H$_2$S enhance the vasorelaxant effect of NO, while others have shown that H$_2$S reduces such activity, perhaps by chemically reacting with NO to form an unidentified nitrosothiol moiety (Whiteman et al. 2006). It is worth noting that H$_2$S at relatively low concentrations enhances vascular tone, most likely by quenching NO and inhibiting endothelial NO synthase (eNOS) (Ali et al. 2006; Kubo et al. 2007). In the present study, we did not observe a significant change in NO metabolite levels in culture. Thus, our data do not implicate a role for NO in H$_2$S-induced proliferation of ICC.

We (Tong et al. 2004) and others (He et al. 2001; Feldstein et al. 2003) have confirmed the discovery of the loss of ICC within a number of human GI motility disorders, including slow transit constipation, diabetic enteropathy, and pseudo-obstruction etc. In several models of ICC injury, the loss of ICC and gut motility is severe, but restoration can take place within weeks. Therefore, the question remains as to what is the origin of newly formed ICC? Several possibilities exist and one reasonable explanation is the proliferation of mature ICC (Huizinga and White 2008). In the present study, we observed that low concentrations of H$_2$S increased mature ICC numbers in vitro. Therefore, H$_2$S may represent a novel pathway that regulates ICC numbers in these disorders. The role of ICC in promoting proliferation in vitro should be explored to develop novel approaches in treating motility disorders of the gut in vivo.

In summary, we demonstrate that exogenously applied H$_2$S promotes the proliferation of mature ICC in vitro at physiologically relevant concentrations. This effect is mediated by phosphorylation of Akt. Our findings also suggest a novel role for H$_2$S as a prosurvival molecule for ICC, and together these results further extend our current understanding of the factors that regulate ICC numbers in the GI tract.

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Competing Interests

The authors declare that they have no conflicts of interest.

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