special communication

Molecular markers expressed in cultured and freshly isolated interstitial cells of Cajal

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Epperson, Anne, William J. Hatton, Brid Callaghan, Philip Doherty, Rebecca L. Walker, Kenton M. Sanders, Sean M. Ward, and Burton Horowitz. Molecular markers expressed in cultured and freshly isolated interstitial cells of Cajal. *Am J Physiol Cell Physiol* 279: C529–C539, 2000.—Located within the tunica muscularis of the gastrointestinal (GI) tract are networks of cells known as interstitial cells of Cajal (ICC). ICC are critical for important basic functions of GI motility such as generation and propagation of slow-wave pacemaker activity and reception of regulatory inputs from the enteric nervous system. We have developed a novel procedure to identify and isolate individual ICC from freshly dispersed cell preparations of the murine small intestine and gastric fundus and to determine differential transcriptional expression We have compared the expression profiles of pacemaker ICC isolated from the murine small intestine (IC-MY) and ICC involved in neurotransmission from the gastric fundus (IC-IM). We have also compared expression profiles between ICC and smooth muscle cells (SMC) and between freshly isolated ICC and cultured ICC. Cultured ICC express smooth muscle myosin, whereas freshly dispersed ICC do not. All cell types express muscarinic receptor types M_2 and M_3, neurokinin receptors NK_1 and NK_3, and inhibitory receptor VIP-1, whereas only cultured ICC and SMC express VIP-2. Both cultured and freshly dispersed IC-IM and IC-MY express the soluble form of stem cell factor, whereas SMC from the gastric fundus express only the membrane-bound form.

gastrointestinal; smooth muscle; c-*kit*; development

INTERSTITIAL CELLS OF CAJAL (ICC) are a unique class of cells found dispersed in gastrointestinal (GI) muscles. These cells are mesenchymal in origin, and as ICC develop, they assume major roles in GI motility (29). ICC are the pacemaker cells responsible for initiating slow-wave activity in GI muscles (15, 36, 44, 45). ICC are also responsible for active propagation of electrical slow waves (13, 24, 33), and these cells also mediate motor inputs from the enteric nervous system (42, 46).

The distribution of ICC varies in the different organs of the GI tract, and anatomic location corresponds to differences in the phenotypes and functions of these cells. For example, in the myenteric plexus region, multipolar ICC (IC-MY) can be found in the rhythmic regions of the stomach, small bowel, and colon of every species studied (24, 28). From studies performed on mice and dogs, IC-MY are thought to generate and propagate electrical slow waves (see Refs. 24 and 28 for comprehensive descriptions of studies performed on IC-MY). In the colons of some species, cells along the border between the circular muscle layer and the submucosa (IC-SM) provide a secondary source of pacemaker activity (40). The types of ICC responsible for pacemaker activity are absent from tonic regions of the GI tract such as the gastric fundus and sphincters (4, 46). ICC in tonic muscles are situated between the muscle fibers in close association with fine nerve processes that innervate the muscle layers. Rhythmic regions of the GI tract also have ICC within the muscle layers, and in some cases these cells are homogenously distributed through both the circular and longitudinal layers (i.e., stomach and colon), whereas in other cases ICC are clustered at specific locations within the circular muscle (i.e., deep muscular plexus of the small intestine). Studies on mice have suggested that "intramuscular" ICC (IC-IM) play a critical role in motor neurotransmission (4, 47). The different functions of the major classes of ICC suggest that there may be differences in gene expression to facilitate their special roles in GI motility. Studies to characterize the differences in ICC have been difficult to perform because of the problems in identifying ICC in cultures and in fresh dispersions of GI muscles.

Freshly dispersed ICC from the submucosal surface of the circular muscle layer of the canine colon are electrically rhythmic (19) and express inward and outward currents that are distinct from those of colonic smooth muscle cells (21). Cultured ICC isolated from the murine small intestine retain the ability to generate electrical rhythmicity, and they may be a powerful model for studying the pacemaker mechanism. Cultured ICC have been identified by the expression of Kit as shown by immunohistochemistry and reverse-transcription polymerase chain reaction (RT-PCR) (18, 32), but it has been far more difficult to positively identify freshly dispersed ICC. It is important to perform basic characterizations of phenotype and function on fresh cells because it is possible that cells change in culture and express genes that do not necessary contribute to the mature phenotype in situ.

In the present study we describe a method to label freshly dispersed ICC with an anti-Kit antibody and to distinguish ICC from other cell types in the preparation. We have selected individual ICC and determined the expression of several molecular components using RT-PCR. We have compared gene expression of IC-MY from the small intestine (pacemaker ICC) and IC-IM from the fundus and compared expression by ICC with neighboring smooth muscle cells (SMC) to begin to develop expression profiles that might explain the functional differences in these cells. We have also compared the expression of key genes in cultured and freshly dispersed ICC to determine whether phenotypic changes occur in culture.

MATERIALS AND METHODS

Tissue dissection. Balb/C mice (20–30 days old for freshly dispersed cells; 9–15 days old for cell cultures) of either sex were anesthetized by CO_2 inhalation and killed by exsanguination following cervical dislocation. The Institutional Animal Use and Care Committee at the University of Nevada approved the use and treatment of animals. The fundus and small intestine (from 1 cm below the pyloric ring to the caecum) were removed and placed in cold Krebs-Ringer buffer (KRB) containing (in mM) 120.4 NaCl, 5.9 KCl, 15.5 $NaHCO_3$, 11.5 glucose, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, and 2.5 $CaCl_2$ (pH 7.3–7.4). Both preparations were opened along the mesenteric border, and the luminal contents were washed with cold KRB. Fundus tissues were pinned to the base of a Sylgard dish, and the mucosa was removed by sharp dissection. Small intestine tissues were pinned down onto a Sylgard dish with the mucosal surface facing downward. The longitudinal muscle and myenteric border were isolated and used as the starting material for the isolation of small intestine myenteric ICC (IC-MY). This tissue dissection was presumably free of another class of ICC occurring at the deep muscular plexus (IC-DMP) in the murine small intestine.

Labeling of ACK monoclonal antibody with Alexa 488 dye. The monoclonal antibody for the Kit protein (ACK2, 1 mg/ml; GIBCO BRL, Gaithersburg, MD) was conjugated to Alexa 488 fluorescent dye (Molecular Probes, Eugene, OR) as per manufacturers' instructions. Briefly, the ACK2 antibody was dialyzed against phosphate-buffered saline (PBS) to remove ammonium ions and primary amines. $NaHCO_3$ (1 M) was subsequently added to the ACK2 protein solution (2 mg/ml) and placed in a vial of Alexa 488 reactive dye for 1 h. The reaction was terminated by the addition of hydroxylamine. The ACK2/Alexa 488 protein solution was then column purified and eluted in $10\times$ PBS (containing 0.1 M potassium phosphate and 1.5 M NaCl, pH 7.2, with sodium azide).

Labeling of freshly dispersed cells with fluorescently labeled c-kit antibody. The fundus and small intestine tissues were incubated for 1 h at 37°C in medium 199 solution (Sigma, St. Louis, MO) containing tetramethylrhodamine isothiocyanate (TRITC)-dextran (Sigma). The samples were rinsed in medium 199 solution, placed in KRB solution containing 5 µg/ml ACK2/Alexa 488 conjugate, and incubated at 4°C for 1 h. The dissected tissues were then equilibrated in Ca^{2+}-free Hanks' solution containing (in mM) 125 NaCl, 5.36 KCl, 15.5 NaOH, 0.336 Na_2PO_4, 0.44 KH_2PO_4, 10 glucose, 2.9 sucrose, and 11 HEPES (pH 7.4) for 1 h. The tissues were then placed overnight at 4°C in enzyme solution containing Ca^{2+}-free Hanks' solution, 1.3 mg/ml collagenase (type II; Worthington), 2 mg/ml bovine serum albumin (Sigma), 2 mg/ml trypsin inhibitor (Sigma), and 0.55 mg/ml adenosine triphosphate at 4°C overnight. The next day, the tissue was incubated at 37°C for 5 min and washed repeatedly with Ca^{2+}-free Hanks' solution. Tissue pieces were triturated to disperse cells.

ICC culture and staining with fluorescently labeled c-kit antibody. Culturing of ICC was performed as described previously (18). Briefly, IC-MY cells were cultured for 3 days in medium containing SMGM (smooth muscle growth medium; Clonetics, San Diego, CA) and supplemented with 5 ng/ml murine stem cell factor. The medium was removed and replaced with medium 199 containing 2 mg/ml TRITC-dextran and then placed at 37°C in a 95% O_2–5% CO_2 incubator for 1 h. The TRITC-dextran solution was removed and rinsed with medium 199. The cultured cells were then placed in medium 199 containing the ACK2/Alexa 488-labeled protein (5 µg/ml) and incubated for 1 h. The cultured cells were then washed three times in medium 199. During the culturing of ICC, SMC were cocultured, and these cells were selected and termed cultured SMC.

Cell selection. Freshly dispersed cells from the fundus and small intestine were placed in separate glass-bottomed dishes on a Nikon Diaphot inverted microscope equipped with fluorescence and phase-contrast optics. The isolated cells were allowed to settle to the bottom of the dish for 10 min, and Hoechst 33342 (Molecular Probes) was added (1 µm/ml) as fluorescent nuclear counterstain. With the use of fluorescence microscopy, cells exhibiting Kit-like immunoreactivity were identified. The optics were switched to phase-contrast, and a micromanipulator (World Precision Instruments) containing a large-diameter micropipette tip was positioned adjacent to the Kit-positive cell. Application of negative pressure resulted in aspiration of a single ICC into the micropipette. Forty to sixty SMC were selected for each RNA preparation as reported previously (6). Each cell selected was expelled into an RNase-free vial, snap frozen in liquid N_2, and stored at -70°C until the time of use. The dishes of cultured cells labeled with TRITC-dextran and Kit/Alexa 488 proteins were examined under fluorescence and phase-contrast optics and selected in a manner similar to that used for the freshly dispersed cells.

Total RNA isolation and RT-PCR. Total RNA was prepared from all tissue and isolated cells by using the SNAP Total RNA isolation kit (Invitrogen, San Diego, CA) as per manufacturer's instructions, including the use of polyinosinic acid (20 µg) as an RNA carrier. First-strand cDNA was prepared from the RNA preparations using the Superscript II Reverse Transcriptase kit (GIBCO BRL), and 500 µg/µl oligo(dT) primers were used to reverse transcribe the RNA

sample. The cDNA reverse transcription product was amplified with specific primers by PCR. PCR was performed in a 25-μl reaction containing 60 mM Tris·HCl (pH 8.5), 1.5 mM MgCl$_2$, 15 mM (NH$_4$)SO$_4$, 1 mM dNTP mix, 10 μM of each primer, cDNA, and 1.0 unit of *Taq* polymerase (Promega, Madison, WI). All reactions were performed in an Applied Biosystems thermal cycler (PerkinElmer, Norwalk, CT) and subjected to hot start of 94°C for 3 min. The majority of the genes were amplified by 33 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 20 s and, finally, an extension step at 72°C for 10 min. In reactions containing c-*kit* and myosin heavy chain primers, the cycling parameters were 40 cycles of 94°C for 15 s, 48°C for 60 s, and 72°C for 90 s, with a final extension step at 72°C for 10 min. Muscarinic receptor types 2 and 3 (M$_2$ and M$_3$, respectively) cycling parameters involved 32 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s, with an extension step at 72°C for 7 min. The amplified products (7 μl) were separated by electrophoresis on a 2% agarose/1× TAE (Tris, acetic acid, EDTA) gel, and the DNA bands were visualized by ethidium bromide staining. The PCR products obtained from RNA isolated from the single-cell preparation were subjected to a second round of amplification and run on a gel.

The following PCR primers were used [the first number range represents the sense bordering nucleotide (nt) positions, and the second number range represents the antisense bordering nucleotide positions; numbers in parentheses are the GenBank accession numbers]: β-actin, nt 2,383–2,402 and 3,071–3,091 (V01217); c-*kit*, nt 2,259–2,283 and 2,873–2,897 (X06182); protein gene product 9.5 (PGP9.5), nt 34–53 and 344–363 (D10699); smooth muscle myosin heavy chain, nt 1,313–1,327 and 1,456–1,474 (J04833); macrosialin (CD68), nt 92–109 and 473–490 (X68273); CD34, nt 798–817 and 1,163–1,182 (S69293); prolyl-4-hydroxylase, nt 68–87 and 431–450 (X06453); mast cell carboxypeptidase A, nt 24–43 and 513–531 (J05118); stem cell factor, nt 220–239 and 528–548 (M57647); transient receptor potential 1 (Trp1), nt 681–700 and 1,212–1,231 (U73625); Trp2, nt 685–704 and 982–1,001 (AF111107); Trp 3, nt 1,483–1,502 and 1,795–1,814 (U47050); Trp 4, nt 2,348–2,367 and 2,596–2,615 (U50922); Trp 5, nt 675–694 and 1,075–1,094 (AF029983); Trp 6, nt 736–758 and 1,129–1,148 (U49069); muscarinic receptor M$_2$, nt 503–524 and 1,290–1,311 (X15264); muscarinic receptor M$_3$, nt 1,438–1,460 and 1,864–1,886 (U29589);

vasoactive intestinal peptide 1 (VIP-1), nt 824–843 and 1,161–1,180 (M86835); VIP-2, nt 167–186 and 421–442 (U09631); neurokinin receptor NK$_1$, nt 325–349 and 621–645 (L27826); and neurokinin receptor NK$_3$, nt 721–740 and 1,279–1,298 (X87823). In every case throughout the study, amplification products of the predicted size for the primer pairs were gel extracted and sequenced to confirm their identity.

Myosin heavy chain is an alternatively spliced transcript, and in smooth muscle both splice forms are expressed (1). In all cDNA preparations, specific primers for β-actin were used as a positive control to assure that each sample was not contaminated with genomic DNA. Primers were designed to span a region of the β-actin gene that encodes an intron; thus, if DNA were contaminating the sample, a 700-bp PCR product would be detected. In addition, negative controls in which reverse transcriptase was not added to the cDNA reactions were included. Any preparations that the controls suggested were contaminated with genomic DNA were discarded. At least three independently isolated ICC or SMC preparations were conducted for each analysis.

RESULTS

Preparation and selection of single dissociated ICC. Whereas previous studies examined isolated ICC on the basis of morphological criteria (19, 21) or studied growing cultures of ICC (18, 32), we selected ICC from fresh enzymatic dispersions of GI muscles by identifying cells with Kit-like immunoreactivity (Kit-LI). Attempts to label cells with Kit-LI after enzymatic dispersion uniformly failed, suggesting that the immunoreactive Kit epitope was damaged or obstructed from antibody binding during digestion. Therefore, we developed a technique that labeled ICC in situ and performed enzymatic dispersion after identification of Kit-LI. These experiments were performed on muscles of the murine gastric fundus (which contain only IC-IM) and the myenteric region of the small intestine (which contain IC-MY). Examination of live-loaded gastric fundus and small intestine tissues before dispersion revealed Kit-LI of the IC-IM network in
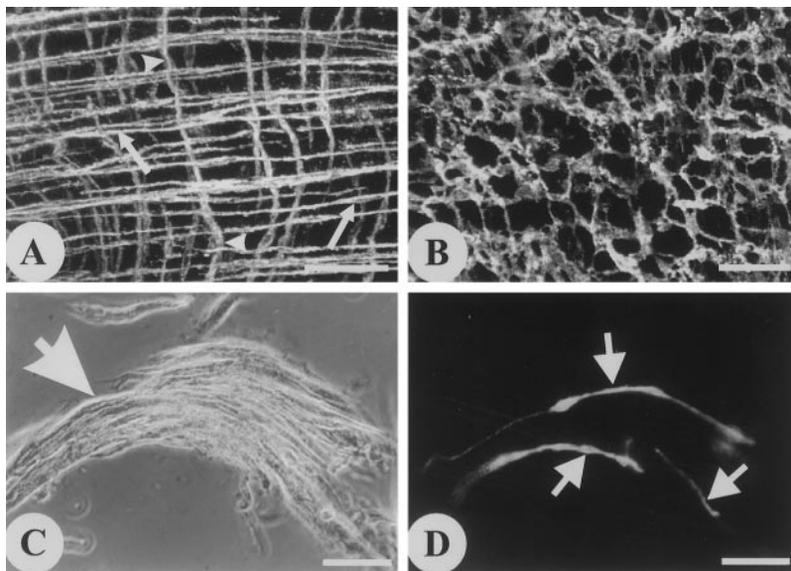

Fig. 1. Fluorescent and phase-contrast images of the murine gastric fundus and small intestine after live-loading with an anti-Kit antibody (ACK2; 5 μg/ml) conjugated with Alexa 488. *A*: a confocal image (digital composite of a *z*-series through 17 μm at 1-μm intervals) of a whole mount preparation of murine gastric fundus showing Kit-like immunoreactive (Kit-LI) labeling of interstitial cells of Cajal (ICC) within the circular (arrows) and longitudinal (arrowheads) muscle layers (IC-IM). *B*: a confocal image (digital composite of a *z*-series through 11 μm at 1-μm intervals) of a whole mount preparation of murine small intestine showing Kit-LI labeling of network of ICC from small intestine at the level of the myenteric plexus (IC-MY). *C*: a phase-contrast photomicrograph showing a group of cells (arrow) after partial enzymatic dispersion of the murine fundus after live-loading with Kit antibody conjugated with Alexa 488. *D*: a fluorescent micrograph of the phase-contrast image of the same field of view as shown in *C*. Within the partially dispersed muscle bundle are several intact IC-IM that still retain Kit-LI (arrows), whereas smooth muscle cells (SMC) are negative for Kit-LI. Scale bars = 50 μm in *A*–*D*.

gastric fundus (Fig. 1*A*) and Kit-LI of the IC-MY network in small intestine (Fig. 1*B*). After dispersion, Kit-LI could be observed in muscle bundles that had not been fully dispersed (Fig. 1, *C* and *D*). The cell dispersion contained cells with Kit-LI and many cells that were not immunoreactive, including SMC (Fig. 2, *A–C*). Examination of the cells with Kit-LI under phase-contrast optics suggested that the cells were either elongated (occasionally with small processes) or round and flat. The latter were found to be macrophages, and these cells could be easily distinguished from ICC by dual labeling with fluorescent dextran (22). Macrophages phagocytosed both the TRITC-dextran and the Kit antibody, and it should be emphasized that Kit-LI was due to phagocytic uptake rather than antibody/antigen binding (Fig. 2, *D–F*). Elongated cells with Kit-LI but no TRITC-dextran uptake (Fig. 2, *G–I*) were selected for mRNA isolation and RT-PCR. SMC were selected from the same dispersed cell preparations for comparative expression studies.

Expression studies were also performed to compare freshly isolated ICC with cultured ICC. Under phase-contrast optics, cultured ICC formed networks with morphologies similar to IC-MY in vivo. After cells had been cultured for 3 days, they were labeled with ACK2/Alexa 488-labeled antibody. Cells organized into IC-MY-like networks had Kit-LI as previously reported (18). Single cells with Kit-LI or small networks were selected for mRNA isolation and RT-PCR.

Expression of marker genes confirming the identity of selected ICC. The GI musculature contains a heterogeneous population of cells, including ICC, SMC, neurons, enteroglia, macrophages, mast cells, fibroblasts, and endothelial cells. Comparative expression studies required selection of specific classes of cells. Therefore, primers were designed to amplify cell-specific markers to test the purity of specific populations of cells. The specific primers we used included c-*kit* (ICC), smooth muscle myosin heavy chain (SMC) (41), PGP9.5 (neurons) (5), macrosialin CD68 (macrophage) (12), CD34 (fibroblast/endothelial cells) (38), propyl 4-hydroxylase (fibroblast) (7), and carboxypeptidase (mast cell) genes (27). Freshly dispersed cells from the murine gastric fundus (IC-IM) and small intestine (IC-MY) with
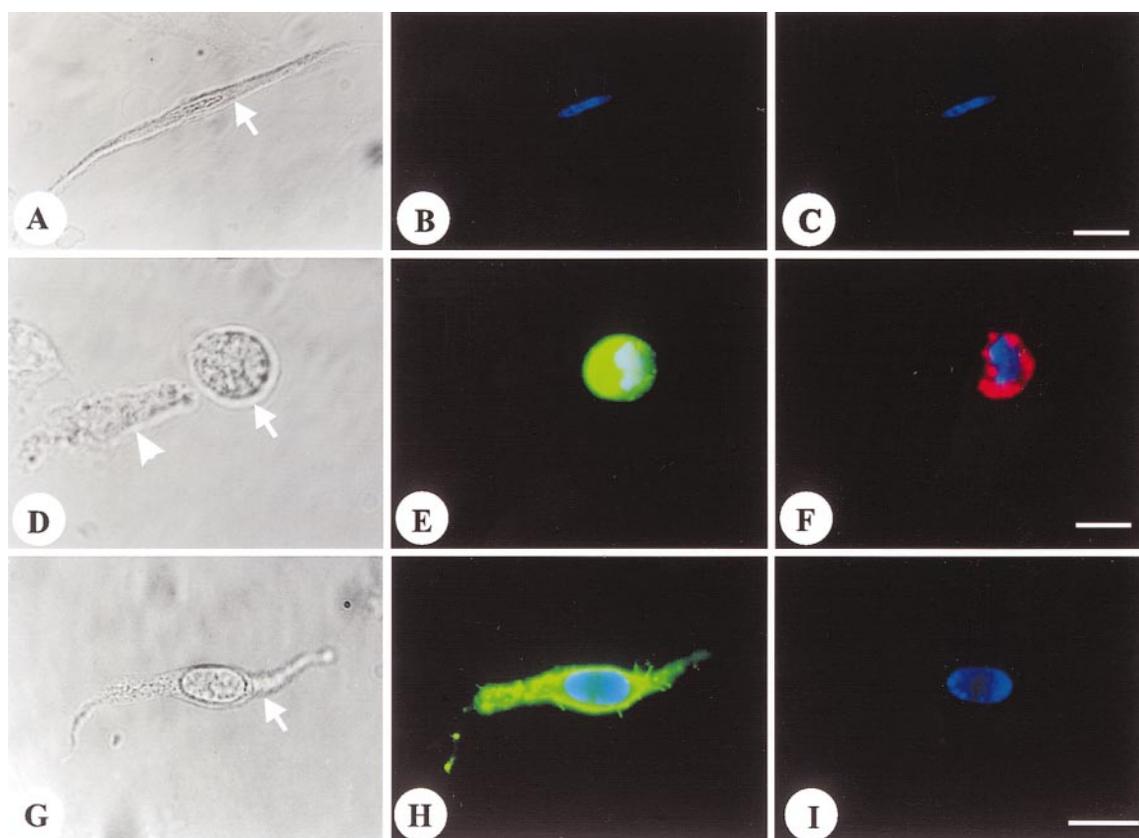

Fig. 2. Phase-contrast and fluorescence photomicrographs of SMC, macrophages, and ICC from enzymatically dispersed murine gastric fundus. SMC and macrophages could be readily distinguished from IC-IM on the basis of their immunoreactivity and phagocytic capability. *A, D,* and *G*: phase-contrast images of typical SMC, macrophages, and ICC, respectively. SMC (*A*, arrow) were immunonegative for Kit (*B*) and did not take up tetramethylrhodamine isothiocyanate (TRITC)-dextran (*C*). Dispersed resident macrophages appeared as rounded cells (*D*, arrow) and were occasionally observed to phagocytose FITC-labeled Kit antibody (*E*, green fluorescence). Macrophages always phagocytosed TRITC-dextran (*F*, red fluorescence). Note that a contracted SMC in *D* (arrowhead) was positive for neither Kit-LI or TRITC-dextran. In contrast, isolated IC-IM (*G*, arrow) were strongly Kit-LI (*H*, green fluorescence) but never phagocytosed TRITC-dextran (*I*). Nuclei of SMC, macrophages, and IC-IM were labeled with Hoechst 33342 (*B, C, E, F, H,* and *I*, blue fluorescence). Scale bars = 20 µm.
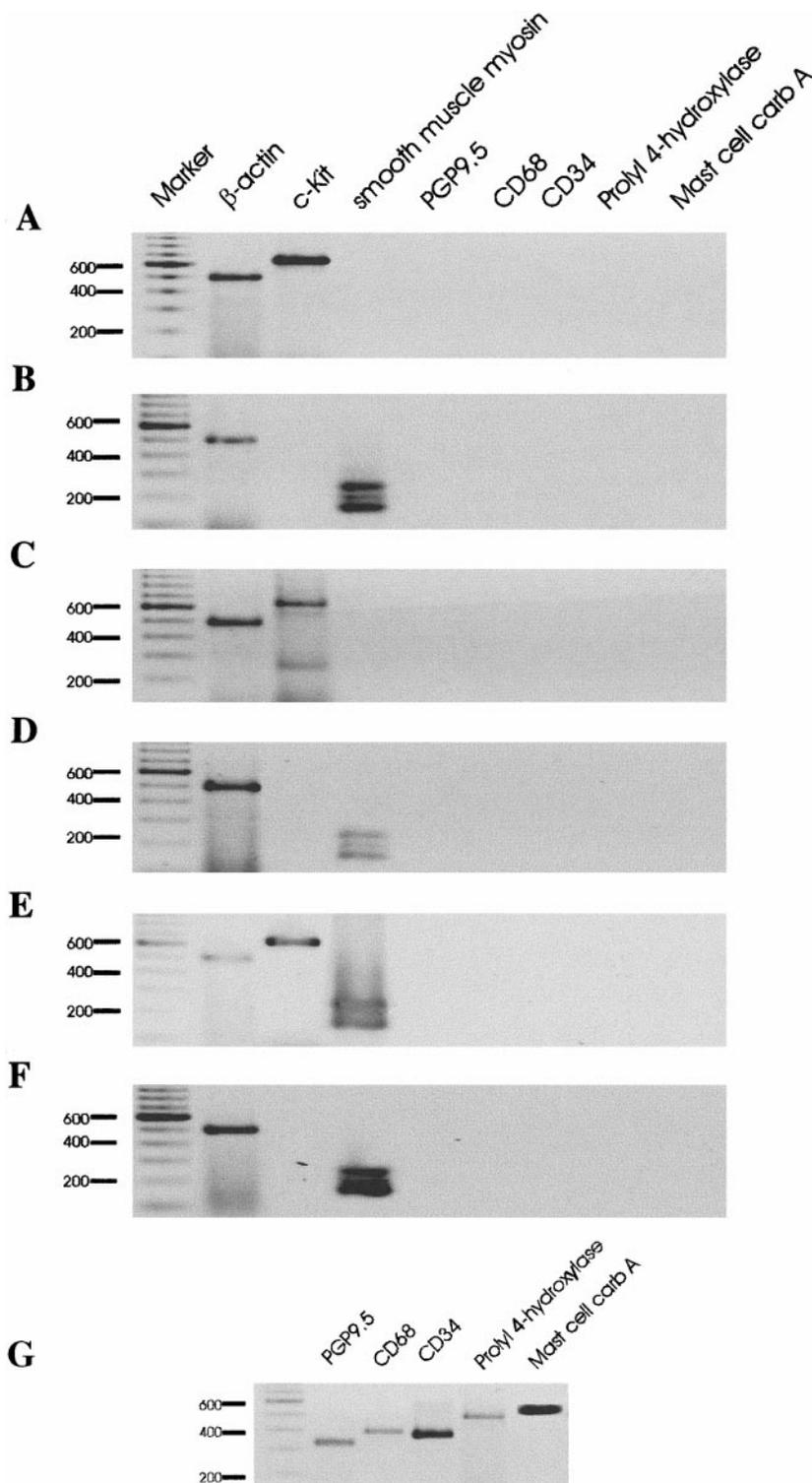
Fig. 3. Transcriptional expression of cell-specific markers in freshly isolated and cultured ICC and SMC. RNA was isolated from freshly dispersed murine fundus IC-IM (*A*) and myocytes (*B*), freshly dispersed small intestine IC-MY (*C*) and myocytes (*D*), and cultured murine small intestine IC-MY (*E*) and myocytes (*F*). As a positive control for primer specificity, murine brain was included (*G*). Primers were designed to amplify genes whose expression is associated with particular cell types, including c-Kit (ICC, 639 bp), smooth muscle myosin heavy chain (SMC, 161 and 200 bp), protein gene product 9.5 (PGP9.5; neurons, 299 bp), CD68 (macrophages, 398 bp), CD34 (fibroblasts, 384 bp), prolyl-4-hydroxylase (fibroblasts, 382 bp), and mast cell carboxypeptidase A (mast cell carb A; mast cells, 508 bp). The PCR products were separated on a 2% agarose/TAE (Tris, acetic acid, EDTA) gel and visualized by ethidium bromide staining. β-Actin was added as a positive control to ensure the integrity of the RNA and the absence of genomic DNA contamination. A 100-bp marker (darker band = 600 bp) was included to illustrate the size of the amplified products.

Kit-LI were positive for c-*kit* mRNA expression and negative for myosin heavy chain, PGP9.5, macrosialin, and tryptase genes (Fig. 3, *A* and *C*). These results confirm that cells with Kit-LI are ICC and are not contaminated by other cells within the suspensions after enzymatic dispersion. Under the same experimental conditions, cells with SMC-like morphology but without Kit-LI did not display c-*kit* PCR products.

These cells expressed smooth muscle myosin heavy chain mRNA and were therefore identified as SMC. Transcripts encoding PGP9.5, macrosialin, and tryptase genes were undetected in SMC (Fig. 3, *B*, *D*, and *F*).

In contrast, cells with Kit-LI selected from cultured cell preparations displayed expression of smooth muscle myosin heavy chain PCR products of the predicted

size [161 and 200 base pairs (bp)] in addition to c-*kit* (Fig. 3*E*). PGP9.5, macrosialin, tryptase, and CD34 PCR products were not detected in cultured ICC.

Expression of molecular components of neurotransmitter pathways. ICC are an important site of innervation of GI muscles by enteric motor neurons (4). In addition, recently ICC have been shown to be critical for cholinergic transmission in the murine fundus (42). We examined the expression of several of the receptors that might be involved in neurotransmission. Previous studies have shown that muscarinic receptors M_2 and M_3 are expressed in muscles of the GI tract (47, 48), and we found that both receptor subtypes were expressed in ICC and SMC from freshly dispersed and cultured preparations (Fig. 4) (48). Similarly, neurokinin receptors NK_1 and NK_3 were found to be expressed in all ICC and SMC preparations tested. Attempts to design specific NK_2 primer pairs failed, and therefore the transcriptional expression of this receptor was not determined. In contrast to the common expression pattern of NK receptors, vasoactive intestinal peptide receptors VIP-1 and VIP-2 were differentially expressed. VIP-1 was expressed in IC-MY and IC-IM, but little or no expression was detected in SMC from the same preparations (Fig. 4, *A–D*). VIP-1 expression was detected in cultured ICC and SMC (Fig. 4, *E* and *F*). VIP-2 expression was not detected in any freshly dispersed ICC or SMC preparations (Fig. 4, *A–D*); however, cultured ICC and SMC displayed expression (Fig. 4, *E* and *F*).

Expression of a membrane conductance target of GI neurotransmitter pathways. Previous studies have identified nonselective cation currents in GI SMC that are activated by excitatory neurotransmitters (20). Another species of nonselective cation current may be the primary pacemaker current (18). Molecular candidates that might encode these currents include members of the Trp (transient receptor potential) gene family. At present there are six known members of this gene family expressed in mammalian tissues (Trp1–Trp6). These genes encode cation channels with similar but not identical properties (2, 49). With the use of primers specific for individual Trp genes 1–6, Trp1 was detected in SMC but not ICC isolated from small intestine (IC-MY) (Fig. 5, *A–B*). In addition, Trp1 expression was detected in SMC but not ICC from cultured preparations (Fig. 5, *E* and *F*). Trp4 and Trp6 were expressed in both IC-MY and SMC. Trp6 expression was also detected in IC-IM and SMC from the gastric fundus. Cultured SMC consistently displayed amplification products for Trp3 and Trp5 (Fig. 5*F*).

Expression of Kit ligand (stem cell factor). Stem cell factor (SCF) is the ligand for the Kit receptor (14) and is necessary for the development of ICC (43). SCF is the product of the *Steel* locus, and alternative splicing of the transcript results in two products that differ by the presence or absence of 28 amino acids near the carboxyl terminus of the protein (8). Whereas both alternative splice forms can encode a soluble form of SCF (due to proteolytic cleavage of the membrane-associated precursor protein), the form missing 28 amino
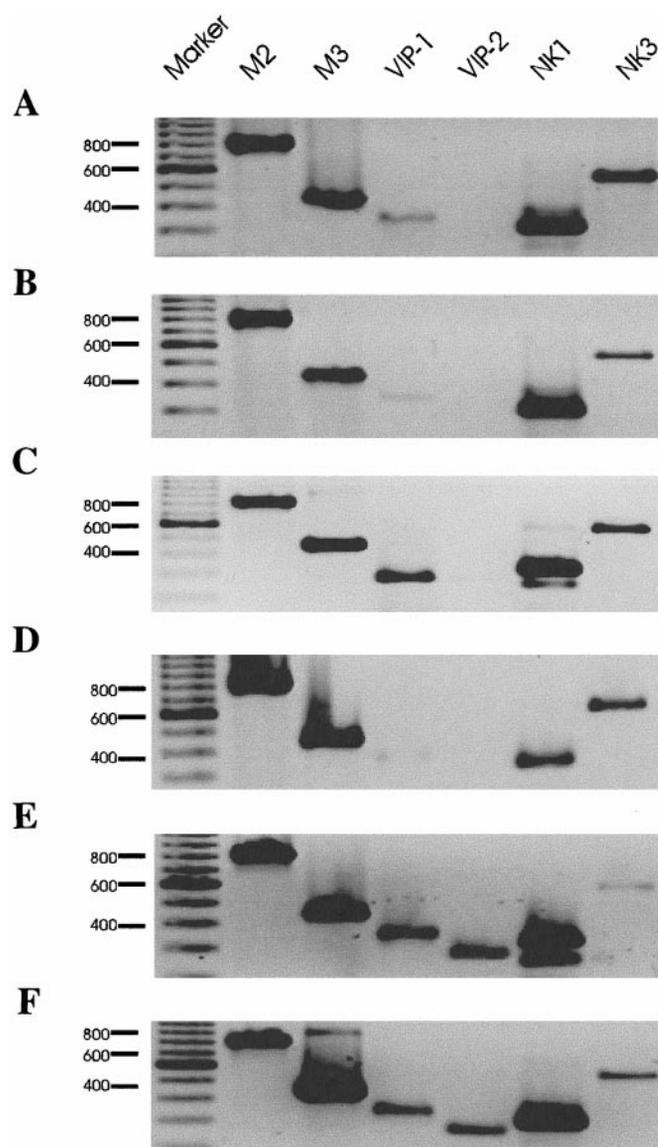

Fig. 4. Detection of neurotransmitter receptors in freshly isolated and cultured ICC and SMC from the murine fundus and small intestine using RT-PCR. RNA was isolated from freshly dispersed murine fundus IC-IM (*A*) and myocytes (*B*), freshly dispersed small intestine IC-MY (*C*) and myocytes (*D*), and cultured murine small intestine IC-MY (*E*) and myocytes (*F*). RT-PCR was performed using gene-specific primers for M_2 (808 bp), M_3 (448 bp), VIP-1 (356 bp), VIP-2 (275 bp), NK_1 (320 bp), and NK_3 (488 bp). Amplified products were separated by electrophoresis on a 2% agarose/TAE gel and then visualized by ethidium bromide staining. A molecular weight marker (darker band = 600 bp) was included to illustrate the size of the PCR fragments.

acids is preferentially membrane bound (because the cleavage site is deleted). We found that freshly dispersed IC-IM express only the soluble form of SCF, whereas SMC from the same preparation express the membrane-bound form (Fig. 6). On the other hand, both IC-MY and SMC from the small intestine only express the soluble form of SCF. Interestingly, these same cells when cultured expressed both splice forms, although the soluble form predominated.

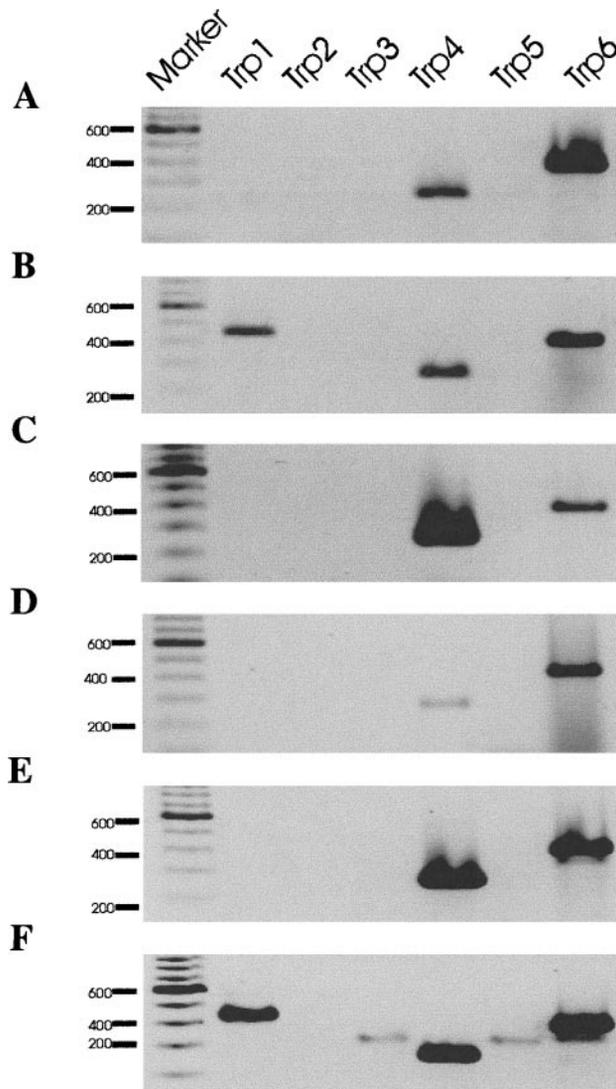Expression results are summarized for all experiments in Table 1.

Fig. 5. Analysis of transient receptor potential (Trp) gene expression in freshly isolated and cultured ICC and SMC from the murine fundus and small intestine. RNA was isolated from freshly dispersed murine fundus IC-IM (A) and myocytes (B), freshly dispersed small intestine IC-MY (C) and myocytes (D), and cultured murine small intestine IC-MY (E) and myocytes (F). RT-PCR was performed using gene-specific primers for Trp1 (550 bp), Trp2 (315 bp), Trp3 (303 bp), Trp4 (250 bp), Trp5 (400 bp), and Trp6 (404 bp). Amplified PCR products were separated by electrophoresis on a 2% agarose/TAE gel and stained with ethidium bromide. A molecular weight marker (darker band = 600 bp) was included to illustrate the size of the PCR fragments.

DISCUSSION

GI muscles are composed of heterogeneous populations of cells, and studies of whole tissues or dispersed cells cannot be used to make conclusions about the gene expression of specific types of cells. Therefore, we developed a method to isolate and select ICC from freshly dispersed cells from GI muscles. Using this procedure, we selected ICC from cell dispersions of murine small intestine and gastric fundus and prepared RNA from single cells to determine the expression of various gene products. These experiments, and future automated molecular studies, will allow devel-

opment of expression profiles for ICC and other cells within the tunica muscularis. This information will be extremely valuable in determining the molecular mechanisms for the unique functions of ICC and the changes that occur in ICC during pathophysiological changes in these cells (see Ref. 29) (37).

Cultures of ICC have emerged as an important new model for studies of the mechanism of electrical rhythmicity in the GI tract because the conductances and "clock" mechanism for slow waves survive cell culturing (18, 32). Caution must be applied to studies of cultured ICC, however, because the artificial environment created during culture conditions might promote changes in phenotype. For example, Thomsen and colleagues (32) concluded that ICC possess a contractile phenotype, because they observed contractions of these cells in culture (32). Our evidence suggests that ICC do not express smooth muscle myosin in situ, but these cells begin to express this gene under culture conditions. These observations may be similar to the changes that occur in situ when ICC are deprived of Kit signaling during development. Under these conditions, ICC take on a smooth muscle-like phenotype including the expression of smooth muscle myosin (see Ref. 34). Thus it is possible that the contractile behavior of ICC is a consequence of cell culture.

Recent studies have suggested that ICC have an important role in neurotransmission (see Refs. 4 and 42). We examined the expression of several neurotransmitter receptors in ICC and compared expression of receptors in IC-MY (pacemaker cells) and IC-IM (mediators of enteric neurotransmission). There was general expression of a variety of receptors that could be involved in enteric neurotransmission. We were unable to detect any differences in the receptor genes examined (muscarinic receptors M_2 and M_3, VIP receptors VIP-1 and VIP-2, neurokinin receptors NK_1 and NK_3), which were selected for study as examples of excitatory and inhibitory receptors, between IC-MY and IC-IM. It is possible that other receptor types could
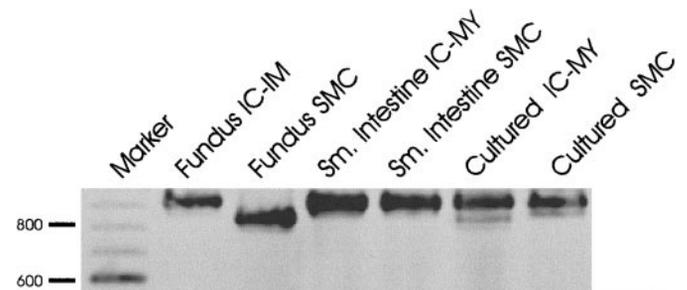

Fig. 6. Stem cell factor (SCF) RNA expression in freshly isolated and cultured ICC and SMC from the murine fundus and small intestine. RT-PCR was performed using gene-specific primers for SCF on RNA prepared from freshly dispersed murine fundus IC-IM and myocytes (fundus SMC), freshly dispersed small intestine IC-MY (sm. intestine IC-MY) and myocytes (sm. intestine SMC), and cultured murine small intestine IC-MY (cultured IC-MY) and myocytes (cultured SMC). The oligonucleotide primers were designed to border an alternative splice site giving rise to 2 amplified products, at 910 bp (SCF-A) and 830 bp (SCF-B), that give rise to the soluble form and membrane-bound form of SCF, respectively (8).

Table 1. *Summary of expression results*

	Fresh IC-MY	Fresh SMC-MY	Fresh IC-IM	Fresh SMC-IM	Cultured IC-MY	Cultured SMC-MY
β-Actin	+	+	+	+	+	+
c-*kit*	+	−	+	−	+	−
Myosin	−	+	−	+	+	+
PGP9.5	−	−	−	−	−	−
CD68	−	−	−	−	−	−
CD34	−	−	−	−	−	−
P4-hydrox	−	−	−	−	−	−
Carb A	−	−	−	−	−	−
M_2	+	+	+	+	+	+
M_3	+	+	+	+	+	+
VIP-1	+	+	+	+	+	+
VIP-2	−	−	−	−	+	+
NK_1	+	+	+	+	+	+
NK_3	+	+	+	+	+	+
Trp1	−	+	−	−	−	+
Trp2	−	−	−	−	−	−
Trp3	−	−	−	−	−	+
Trp4	+	+	+	+	+	+
Trp5	−	−	−	−	−	+
Trp6	+	+	+	+	+	+
SCF-A	+	−	+	+	+	+
SCF-B	−	+	−	−	+	+

Experiments were performed with freshly dispersed and cultured interstitial cells of Cajal from the small intestine (IC-MY) or the gastric fundus (IC-IM) as well as with smooth muscle cells (SMC) from the same sources. PGP9.5, protein gene product 9.5; P4-hydrox, prolyl-4-hydroxylase; Carb A, mast cell carboxypeptidase A; M_2 and M_3, muscarinic receptor types 2 and 3; VIP-1 and -2, vasoactive intestinal peptides 1 and 2; NK_1 and NK_2, neurokinin receptor types 1 and 2; Trp1–Trp6, transient receptor potentials 1–6; SCF-A and SCF-B, stem cell factors A and B.

be differentially expressed in the different classes of ICC or that the location of ICC (e.g., IC-IM associated with enteric nerves) may determine the function of particular ICC. Both cultured ICC and SMC expressed VIP-1 and VIP-2, in contrast to their freshly dispersed counterparts, illustrating another subtle difference in expression pattern between cultured and freshly dispersed cells. Interestingly, VIP-2 expression was not detected in freshly dispersed and selected IC-MY or IC-IM or in SMC from those preparations. This is in contrast to the findings of other investigators who observed VIP-2 expression in guinea pig gastric and tenia coli SMC (31). This study used guinea pig gastric muscle, which indicates a species difference, and the authors did not isolate SMC but instead examined RNA prepared from a cell dispersion that may contain other, non-SMC types. Characterization of neurokinin receptors was interesting in that we found expression patterns different from those that have been reported using neurokinin receptor-specific antibodies and immunofluorescence. Several labs have reported NK_1 receptors in ICC and enteric neurons, but not in SMC (10, 26, 30). NK_3 are thought to be mainly neural receptors expressed by myenteric and submucosal neurons (10). Our data suggest that expression of subpopulations of neurokinin receptors may not be as cell-specific as previously believed.

Candidates for the nonselective cation channels that are coupled to muscarinic or neurokinin receptors include members of the Trp gene family. Trp1, Trp4, and Trp5 are activated by Ca^{2+} store depletion and may constitute store-operated or capacitive Ca^{2+} entry channels (2, 50). However, Trp3 and Trp6 are not sensitive to Ca^{2+} store depletion and are activated by the phospholipase C pathway (3, 50) or directly by diacylglycerol (11). Electrophysiological studies show that Trp3 and Trp6 encode nonselective cation channels with relatively large single-channel conductance [60 pS for Trp3 (16)]. However, expression of human recombinant Trp3 (hTrp3) in HEK-293 cells resulted in a 17-pS channel (17). We have recently cloned and expressed Trp6 from mouse GI SMC. In SF9 insect cells, Trp6 expression results in a nonselective cation current that is blocked by La^{3+} and Gd^{3+} and is not sensitive to store depletion (39). Trp gene products are expressed in ICC and SMC from freshly dispersed and cultured preparations. The most abundant forms were Trp4 and Trp6. Trp6 can be activated by muscarinic stimulation, and therefore this channel may contribute to the response to excitatory neurotransmission. Trp4, on the other hand, has been shown to be sensitive to Ca^{2+} store depletion and shows selectivity for divalent (Ba^{2+} and Ca^{2+}) over monovalent cations (25). A caveat to describing properties for these channels from expression in heterologous systems is that they can form heteromeric channels with properties different from those of the channels expressed alone (9). However, identifying which of these channels are expressed in ICC and SMC will allow more thorough analysis of their properties and potential roles. Trp1 was expressed in SMC from the gastric fundus and was not expressed in ICC. Trp1, like Trp4, appears to be a Ca^{2+} store depletion-operated channel and may be involved in capacitive Ca^{2+} entry in these cells. It is interesting that Trp1 expression could only be detected in SMC from the gastric fundus and not from the small intes-

tine. Cultured SMC from the small intestine were found to express Trp1, but these cells also began to express Trp3 and Trp5. The significance of the proliferation of Trp isoforms in culture is not clear.

ICC development depends on signaling via the Kit tyrosine kinase pathway (see Refs. 35 and 36). The natural ligand for Kit is SCF (see Ref. 14). We examined SCF expression in ICC and SMC to learn more about the microenvironment that might contribute to the development of ICC. ICC and SMC were both found to express SCF. This suggests that interactions between these cells may be important during the development of the ICC phenotype. IC-IM expressed only the soluble form of SCF, whereas SMC from the same preparations of gastric fundus expressed only the membrane-bound form. Because membrane-bound SCF expression is required for proper development of ICC (23, 43), it is possible that cell-to-cell contact between ICC and SMC is needed in the fundus for the development of ICC. Continued interactions between these cell types may serve to maintain the ICC phenotype. Expression patterns of SCF in the small intestine differed from those in the fundus. In the small bowel, ICC and SMC expressed only the soluble form of SCF. It is possible that other cell types expressing membrane-bound SCF stimulate the development of ICC in this tissue or that immature Kit-positive cells or SMC may express the membrane-bound form of SCF during a developmental window. Differential expression of SCF in small bowel versus gastric fundus may explain why the distribution of ICC is so different in these organs. In the fundus, ICC form a discontinuous network with IC-IM distributed throughout the circular and longitudinal muscle layers (see Ref. 4). This distribution may be promoted by the expression of membrane-bound SCF by SMC (i.e., Kit-positive mesenchymal cells may find appropriate stimulation of Kit throughout the tunica muscularis). In the small intestine, IC-MY are distributed within the myenteric plexus region and IC-DMP are distributed in a thin surface at the level of the deep muscular plexus. The reasons why ICC are concentrated in these regions are currently unknown. Future studies will need to concentrate on the expression of isoforms of SCF in the regions of the tunica muscularis where ICC develop to address the question of why ICC develop at specific sites. Microdissection techniques and the cell selection methods developed in this study will facilitate these experiments.

Cultured ICC differ from freshly dispersed ICC derived from the same tissue (IC-MY). Cultured cells express both splice forms of SCF, whereas freshly dispersed cells only express the soluble form of SCF at the transcriptional level. This observation may explain why IC-MY have a high propensity to form networks of cells in culture conditions (see Ref. 18). The IC-MY phenotype may require cell-to-cell interactions. This observation may also offer a clue for future experiments in which the specific isoforms of SCF are characterized during development. It is possible that immature IC-MY could express membrane-bound SCF, which might promote the formation of ICC networks. After development of ICC networks, expression patterns may change. It is interesting that ICC and SMC, which may have a common mesenchymal cell origin (see Ref. 36), both begin to express the membrane-bound splice variant of SCF in culture. This may indicate that both cells return to a similar primitive, proliferative state under culture conditions. The common expression of smooth muscle myosin in ICC and SMC is another indication that the two phenotypes become more similar in culture.

In summary, we have developed a technique to isolate freshly dispersed ICC and determine transcriptional expression in these cells. Several interesting differences in expression were detected between cultured and freshly dispersed ICC from the same source. IC-MY and IC-IM display remarkably similar expression patterns for the gene products tested, and differences in functions of these two classes of ICC may be more related to their position in the tissue than to phenotypic properties. A caveat that must be included in this discussion concerns the molecular nature of the findings. Transcriptional expression indicates the propensity for functional expression of a gene product; however, the functional evidence and role has not been established. A coordination of this technique and more traditional biochemical and electrophysiological approaches to studying these cells will lead to an effective analysis of these specialized cells. Further analysis of other gene products and the potential for manipulation of freshly dispersed ICC are exciting prospects resulting from the utilization of this labeling and isolation technology.

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