Immunomagnetic enrichment of interstitial cells of Cajal

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Ördög, Tamás, Doug Redelman, Nancy N. Horowitz, and Kenton M. Sanders. Immunomagnetic enrichment of interstitial cells of Cajal. Am J Physiol Gastrointest Liver Physiol 286: G351–G360, 2004.—Disruptions of networks of interstitial cells of Cajal (ICC), gastrointestinal pacemakers and mediators of neurotransmission, can lead to disordered phasic contractions and peristalsis by reducing and uncoupling electrical slow waves. However, detailed analysis of the ICC network behavior has been hampered by their scarcity, limited accessibility in intact tissues, and contamination with other cell types in culture. Our goal was to develop a simple technique to purify ICC from murine gastrointestinal muscles for functional studies. We identified ICC in live small intestinal muscles or primary cell cultures by Kit immunoreactivity using fluorescent antibodies. Because this technique also labels resident macrophages nonspecifically, parallel studies were performed in which nonfluorescent Kit antibodies and macrophages labeled with fluorescent dextran were used for subtractive analysis of ICC. In both groups, Kit-positive cells were tagged with superparamagnetic antibodies and sorted on magnetic columns. Efficiency was assessed by flow cytometry. ICC enrichment from primary cultures and freshly dissociated tissues was ~63-fold and ~8-fold, respectively. Unlike the cells derived directly from tissues, cells sorted from cultures frequently yielded extensive, nearly homogenous ICC networks on reseeding. Monitoring oscillations in mitochondrial Ca²⁺ or membrane potential by imaging revealed spontaneous rhythmicity in these networks. Cells that did not bind to the columns yielded cultures that were depleted of ICC and dominated by smooth muscle cells. In conclusion, immunomagnetic sorting of primary cultures of ICC results in relatively homogenous, functional ICC networks. This technique is less suitable for obtaining ICC from freshly dispersed cells.

The discovery that ICC can be identified by expression of the receptor tyrosine kinase Kit [stem cell factor (SCF) receptor, CD117] (13, 21, 39, 42) has led to several studies on the morphology of ICC networks in human GI disorders. Damages to ICC networks have been described in congenital and acquired GI disorders including anorectal malformations, Hirschsprung disease, infantile pyloric stenosis, inflammatory bowel disease, diabetic gastroenteropathy, stromal tumors, and para-neoplastic, idiopathic, or “functional” disorders (11, 20, 23, 27, 28, 32, 41). Many of these changes have also been demonstrated in animal models (2, 3, 19, 24, 25, 34), which provide an exciting opportunity to study the mechanisms and consequences of ICC loss in these diseases. For example, we have demonstrated that disruptions in gastric ICC networks resulting from impaired Kit signaling (25, 26) can lead to electrical arrhythmias by interfering with pacemaker entrainment and by remodeling of the ICC pacemaker apparatus (24). Since similar losses of ICC also occur in motility disorders, such as diabetic gastroenteropathies (11, 23, 25), which are also frequently associated with disturbed electrical pacemaking (15), it is likely that ICC damage and electrical abnormalities in diabetes and other disorders are causally related.

Pacemaker entrainment (i.e., the transformation of individual cells into a large-scale functional network) is critical for the proper function of ICC in vivo. However, analysis of the network behavior of ICC has been difficult because of their scarcity, their limited accessibility in intact tissues, and the random nature of their networks in culture. ICC have been successfully studied by electrophysiological and imaging techniques in primary cultures (16, 17, 29, 36, 37, 44) and even in situ (6, 30, 46). However, these techniques can only target a very limited number of ICC confined to small parts of large networks and therefore cannot reveal interactions that occur on a larger scale. Recently we have attempted to study pacemaker entrainment by coculturing cells from gastric corpus and antrum following labeling with cell-tracking dyes (24). Although this approach can yield interconnected clusters of corpus and antrum ICC, their occurrence was random. Purification and coculturing of functionally active ICC from various anatomic locations that normally have different intrinsic slow-wave frequencies would permit engineering of specialized interconnected networks and would also allow manipulation of the pattern and size of these networks for experimental purposes. Such engineered ICC networks could provide insights into the role of ICC-ICC interactions in slow-wave arrhythmias.

In the present work, we have evaluated immunomagnetic sorting (MACS), a bench-top technique, for obtaining enriched populations of ICC to use for functional studies. MACS may...
be useful for obtaining functionally active ICC because 1) the technique permits simultaneous sorting of large numbers of cells, which is an advantage when purifying rare cells, and 2) it facilitates the recovery of intact cells with minimal stress during sorting (22).

MATERIALS AND METHODS

Animals. Nine- to thirteen-day-old BALB/c mice were obtained from breeder pairs purchased from Simonsen Laboratories (Gilroy, CA). The animals were anesthetized by isoflurane inhalation (Aer- rane; Baxter Healthcare, Deerfield, IL) and killed by decapitation. Mice were maintained and the experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the American Physiological Society’s Guiding Principles in the Care and Use of Animals. All protocols were approved by the Institutional Animal Use and Care Committee at the University of Nevada, Reno.

Tissue preparation. All steps were performed in Krebs-Ringer-bicarbonate solution (see Solutions). The buffer was kept in melting ice and changed frequently during the dissection. Small intestines (jejunum and ileum) were excised and opened along the mesentery, and their contents were washed away with buffer. The mucosa and submucosa were removed by peeling, and only the tunica muscularis of the entire jejunum and ileum was used.

Primary cell cultures. Small intestinal muscles consisting of the entire jejunum and ileum from 9- to 13-day-old mice (2 per experiment) were minced, equilibrated in Ca2+-free Hanks’ solution (see Solutions) for 15 min, and incubated without agitation at 37°C for 23 min in an enzyme solution containing collagenase (1.3 mg/ml Worthington Type II; Worthington Biochemical, Freehold, NJ), BSA (2 mg/ml), trypsin inhibitor (2 mg/ml), and ATP (0.27 mg/ml) (all from Sigma, St. Louis, MO) in Ca2+-free Hanks’ solution. After three washes, the tissues were triturated through a series of three blunt pipettes of decreasing tip diameter. The resulting cell suspension was mixed and cultured in sterile, collagen-coated (2.5 μg murine collagen/ml; Collaborative Biomedical Products, Bedford, MA) T-25 tissue culture flasks (4 per experiment). Cells were cultured at 37°C in a 5% CO2 incubator in Smooth Muscle Growth Medium 2 (BioWhittaker, Walkersville, MD) supplemented with an antibiotic/antimycotic mixture (200 U/ml penicillin, 200 μg/ml streptomycin, and 0.5 μg/ml amphotericin B; Invitrogen, Carlsbad, CA) and soluble murine SCF (25 ng/ml; Sigma). The medium was changed after 24 h to Smooth Muscle Growth Medium 2 containing SCF but no antibiotic/antimycotic. Cultures were used after they had become confluent (~6–8 days after plating) because ICC display stronger Kit immunoreactivity in the presence of other cell types.

Enrichment of ICC by MACS. We based our approach on the detection of the ICC marker Kit with extracellularly reacting rat monoclonal (IgG2b) antibodies (ACK2). Because the epitope recognized by ACK2 is altered or masked by collagenase, ICC labeling had to be performed in the tissues or cell cultures before dispersion (7, 10). Because this technique is relatively simple and has been used by many groups (7, 10), we have designed the following subtractive approach to assess specific ICC enrichment (Fig. 1).

Enrichment of ICC in freshly dispersed cell suspensions. Small intestines (jejunum, ileum) from two 9- to 13-day-old BALB/c mice were used in each experiment. After being peeled, the tissues were halved along their longitudinal axes and the opposite halves were recombined to compensate for potential differences between animals. In one of these recombined tissues, ICC (and macrophages) were labeled with ACK2 that had been conjugated with the fluorescent dye Alexa Fluor 488 (AF 488) using the Alexa Fluor 488 protein labeling kit from Molecular Probes (Eugene, OR). AF 488-ACK2 conjugates were diluted to 10 μg/ml with Ca2+-containing, HEPES-buffered physiological salt solution (see Solutions) and applied at 4°C for 3 h. In the parallel samples, macrophages were first selectively labeled with FITC-dextran [70 kDa, 1.3 mg/ml in phenol red-free medium 199 (all from Sigma)] (7) by incubating the tissues at 37°C for 1 h in a 5% CO2 incubator. After being washed with cold physiological salt solution, ICC (and macrophages) were labeled with nonfluorescent ACK2 (10 μg/ml) as described above. This way the same cells (ICC + macrophages) were identified in both tissues for MACS, but only macrophages had fluorescent labels in the second tissue, whereas both ICC and macrophages were tagged with a fluorescent dye in the first one (Fig. 1). After being labeled, both tissues were washed and equilibrated with cold Ca2+-free Hanks’ solution, transferred into collagenase solution (see Primary cell cultures), and incubated, without stirring, at 4°C overnight. Then the tissues were washed to 37°C for 7 min for the final digestion. After three washes, the tissues were triturated through a series of three blunt pipettes of decreasing tip diameter. The resulting cell suspensions were sedimented by centrifugation (300 g; 5 min; 4°C) and washed with 4 ml of carefully degassed, ice-cold, Ca2+-free Hanks’ solution containing 2% BSA and 2 mM EDTA (sorting buffer). ACK2 was then reacted with goat F(ab’)2 anti-rat IgG conjugated with superparamagnetic beads (Miltenyi Biotec, Auburn, CA; 1:5 in sorting buffer; 4°C for 15 min). The average diameter of these magnetic microparticles is ~50 nm; they are biodegradable and do not influence cell function and viability (22). After being washed, the labeled cells were resuspended in 1 ml sorting buffer and passed through prewetted polyester filters (30 μm mesh size; Miltenyi), which were then washed three times with 1 ml sorting buffer. The volume of the flowthrough was adjusted to 4 ml, and 0.5 ml of this suspension was saved and diluted to 1 ml (unsorted group). The remaining 3.5 ml were then passed through prewetted MS magnetic columns placed in a strong magnet (MiniMACS; all from Miltenyi). Cells not retained on the columns by the magnet were washed three times with 0.5 ml sorting buffer. After the final volume was adjusted to 5 ml, this fraction was saved as the MACS– group. The columns were then removed from the magnet, and the retained cells were flushed out with 1 ml sorting buffer (MACS+ group). Dead cells in all three groups were labeled with propidium iodide (PI; 1.5 μg/ml; BioSci Controls, Grass Valley, CA) and live (PI–) cells labeled with AF 488-ACK2 or FITC-dextran were analyzed by flow cytometry (FCM; see FCM analysis).
Enrichment of ICC from primary cell cultures. Parallel cultures (2
T-25 flasks, derived from 1 small intestine per group) were labeled for
subtractive analysis as described above (Fig. 1), except that the native
or fluorescent ACK2 was applied for 1 h only. After being labeled,
cells were washed and equilibrated with cold, Ca²⁺-free Hank's
solution and then detached with an enzyme solution containing 0.05%
trypsin and 0.53 mM EDTA-4Na (Invitrogen). The reaction was
monitored under the microscope at room temperature and stopped by
adding Ca²⁺-free Hank's solution containing 5% FBS. The dissociated
cells were then washed, reacted with secondary antibodies
conjugated with magnetic beads, and sorted on magnetic columns as
described above. After being sorted, MACS-positive cells were either used
for FCM or were plated onto sterile 35-mm tissue culture dishes, the
bottoms of which had been replaced by No. 1 glass coverslips and
coated with murine collagen as described in Primary cell cultures.

FCM analysis. Double-labeled cell suspensions (AF 488-ACK2 or
FITC-dextran and PI) were analyzed by a Beckman-Coulter XL/
MCL flow cytometer equipped with an Ar ion laser (excitation
wavelength, 488 nm), a photodiode to measure light scattered at low
forward angles (forward scatter), and photomultiplier tubes to mea-
sure orthogonally scattered light (side scatter) plus four wavelengths of
fluorescence: 525 nm (used for the detection of AF 488-ACK2,
emission maximum (Eₘ), 519 nm; or for the detection of FITC-
dextran, Eₘ = 518 nm); 575 nm (used for PI, Eₘ = 617
nm); and 675 nm (unused). Cells were detected by triggering on
forward-scatter signals and data files of ≥20,000 cells were collected
with the Coulter System II acquisition software. These combina-
tions of fluorescence labels did not require any compensation to
resolve the green and red emissions. Listmode data files were ana-
lyzed with SuperCyt Analyst (Sierra Cytometry) software. Regions
were created to define cell clusters with single or double fluorescent
labeling. Dead (PI⁺) cells were excluded from further analysis, and
the proportion of the labeled, live cells was expressed as the percent-
age of the total live cell count reported by this technique. The absolute
number of labeled cells that did not take up PI was calculated from the
number of cells with the appropriate fluorescence parameters over a
certain time, the flow rate (~25 μl/min; verified by using fluorescent
beads at known concentrations), and the total volume of the cell
suspensions. Proper instrument operation was verified by examining
standard reference beads.

Morphological imaging. Phase-contrast micrographs of cell cul-
tures were taken on Kodak Tmax 400 or P3200 black-and-white film
with a Nikon F2 camera mounted on a Nikon Diaphot microscope
equipped with a ×100/0.25 numerical aperture (NA) lens (Nikon
Instruments, Melville, NY). Live-labeled cells or tissues (grown in
glass-bottom dishes) were fixed with 4% paraformaldehyde-saline
(pH 7.4; 10 min at room temperature) for verifica-
tion of labeling by confocal imaging. Labeled specimens were examined with a Bio-Rad
MRC 600 confocal microscope (Hercules, CA) equipped with an
Ar-Kr laser and coupled to a Nikon Diaphot inverted microscope.
Images were acquired with Nikon Fluoar ×40/1.30 NA or Nikon
PlanApo ×60/1.40 NA oil immersion objectives. The confocal mi-
crographs in this manuscript are digital composites of Z-series scans
constructed with CoMOS software (version 7.0a; Bio-Rad).

Fluorescent imaging of pacemaker activity in purified ICC. To
monitor oscillations in mitochondrial Ca²⁺ concentration in purified
and recultured ICC (24, 44), cells were loaded with 4.4 μmol/l
reduced rhod-2 AM, prepared according to the manufacturer’s rec-
ommendations (Molecular Probes), in physiological salt solution
containing 5% FBS to 1 h at 4°C. Loaded cells were cultured for an
additional ~18 h in phenol red-free medium 199 containing 5% FBS
to ensure complete elimination of the dye from the cytoplasm (24, 40).
Previously, we have verified the mitochondrial localization of this dye
by coloading mitochondria with MitoTracker Green FM (24, 44).
Time-series experiments were performed in physiological salt
solution warmed to 29 ± 0.5°C. Mitochondrial Ca²⁺ oscillations associ-
ated with electrical pacemaking in ICC were monitored by using the
line scan option of the Bio-Rad MRC 600 (acquisition rate, 4.2 Hz;
excitation wavelength, 568 nm) as described previously (24, 44).
Traces were smoothed offline by adjacent averaging using Microcal

In separate experiments, we monitored electrical pacemaker activity
in purified, cultured ICC with tetramethylrhodamine methyl ester
(TMTRM; Molecular Probes), a fluorescent, lipophilic, cationic dye
that can undergo potential-dependent redistribution across cellular and
intracellular membranes (8). TMTRM was dissolved in physiological
salt solution and added to the cells at a concentration of 100 nm/l.
Time series experiments were performed with confocal line-scanning
microscopy as described for rhod-2 above.

Solutions. Concentrations are given in millimoles per liter. Kresb-
Ringer-bicarbonate solution contained 123 NaCl, 5.9 KCl, 2.5
CaCl₂, 1.2 MgCl₂, 15.5 NaHCO₃, 1.2 NaH₂PO₄, and 11.5 glucose, pH
7.3–7.4 when bubbled with 97% O₂-3% CO₂. Ca²⁺-free Hank's
solution contained 125 NaCl, 5.36 KCl, 15.5 NaHCO₃, 0.336
Na₂HPO₄, 0.44 KH₂PO₄, 10 glucose, 2.9 sucrose, and 11 HEPES,
adjusted to pH 7.2 with NaOH. Sorting buffer contained Ca²⁺-free
Hanks solution, 2% BSA, and 2 mM EDTA. Physiological salt
solution contained 135 NaCl, 5 KCl, 2 CaCl₂, 1.2 MgCl₂, 10 glucose,
and 10 HEPES, adjusted to pH 7.4 with Tris.

Statistical analyses. The SigmaStat statistical software for Win-
dows version 2.03 (SPSS Science, Chicago, IL) was used for all
statistical analyses. Data are expressed as means ± SE, and n signifies
the number of independent experiments in the tests. The frequency of
rhod-2 and TMTRM oscillations was calculated from the mean inter-
event interval for the particular recording. Before tests of significance
were performed, data were examined for normality and equal variance
to determine whether parametric or nonparametric tests should be
employed. Percentage data were transformed [arcsin(√x)] before statistical analysis. One-way ANOVA followed by all-pairwise mul-
tiple comparison (Tukey test) was used for statistical comparisons. A
probability value of P < 0.05 was used as a cutoff for statistical
significance in all procedures.

RESULTS AND DISCUSSION

We attempted to enrich ICC freshly dispersed from small
intestinal muscles of juvenile (9- to 13-day old) mice or from
primary cell cultures derived from such tissues by MACS, a
simple bench-top technique. Quantitative assessment of the
efficacy of the ICC enrichment was performed by FCM. ICC
were labeled with MACS with ACK2, a rat monoclonal antibody
that recognizes the ICC marker Kit (21, 26, 32, 42), and goat
F(ab')₂ anti-rat IgG secondary antibodies conjugated with
superparamagnetic beads. Since this technique cannot distin-
guish between pacemaker ICC and ICC that mediate neuro-
muscular neurotransmission, in this exploratory work we
treated ICC as a homogenous population. Live immunostaining
with fluorescent conjugates of ACK2 resulted in uniform,
quantitative labeling of ICC networks both in intact tunica
muscularis tissues (Fig. 2A) and in primary cell cultures (Fig.
2, D and E). As reported before (7, 10), this technique also
labeled resident macrophages nonspecifically (Fig. 2, A–C
and F–H), which unavoidably took up the ICC label and accumu-
lated it in intracellular compartments, although binding to Fc
receptors could not be excluded, especially in cultures (Fig. 2,
F–H). Therefore, we identified macrophages with a second
marker, fluorescent dextran, which quantitatively stained these
cells (Fig. 2, B, C, G, and H). Unfortunately, the dye combi-
nation illustrated in Fig. 2, A–C and F–H was not suitable for
FCM analysis with our Beckman-Coulter XL/MCL because it
has only one excitation wavelength available (488 nm) and
cannot excite Alexa Fluor 594. We also wanted to restrict our
Fig. 2. Vital immunolabeling of ICC in tissues and cell cultures and nonspecific staining of resident macrophages. A–C: confocal images of small intestinal muscles from a juvenile BALB/c mouse stained live with the ICC label Alexa Fluor 594 (AF 594)-ACK2 (A) and the macrophage label FITC-dextran (B). C: composite image. ICC networks between and within muscle layers were labeled throughout the tissue (A). Nonspecific uptake of the fluorescent antibody by macrophages was revealed by colabeling with FITC-dextran as shown in B and by the yellow color in C, D and E: confocal images of ICC networks in primary cultures of gastrointestinal muscles stained with Alexa Fluor 488 (AF 488)-ACK2. D: ICC in a monolayer culture. E: ICC within small clumps consisting mainly of smooth muscle cells. Note that Kit-like immunoreactivity was stronger in confluent and overgrown cultures (E) than in monolayers (D). F–H: a macrophage in a primary cell culture prepared from small intestinal muscles. F: nonspecific uptake of AF 594-ACK2. G: accumulation of FITC-dextran in the same cell. H: composite image. Note that the labeled ACK2 and dextran were localized in different intracellular compartments. Some of the staining with AF 594-ACK2 appeared to be associated with the cell membrane. Scale bars in C, E, and H apply to all images in the corresponding row. I: quantitative analysis by FCM of cells dispersed from small intestinal muscles vitally labeled with AF 488-ACK2 (horizontal axis). Live cells were identified by their ability to exclude PI (vertical axis). Each cell in the 2-dimensional logarithmic plot of fluorescence intensities is represented by a single dot. Pseudocolor signifies frequencies of cells with similar fluorescence parameters. Live cells with green (AF 488) fluorescence (bottom right quadrant) were clearly distinguishable from unlabeled live (bottom left quadrant) and dead cells (top quadrants).
analyses to live cells, which required the use of PI as a second label to identify dead cells (Fig. 2I). Because multicolor applications and the use of secondary antibodies may raise additional technical problems (color bleedover, unexpected uptake by otherwise irrelevant cell types, etc.), in this study we used AF 488-ACK2 and FITC-dextran, which have nearly identical spectral properties, and assessed the numbers of live ICC before and after MACS by using a subtractive approach (Figs. 1, 3, and 4; see MATERIALS AND METHODS for details). Briefly, in one sample we labeled both ICC and macrophages with AF 488-ACK2, which served both as the primary antibody for MACS and as a fluorescent tag for FCM. In a parallel sample, we labeled macrophages only with FITC-dextran for FCM while sorting both ICC and macrophages with the aid of nonfluorescent ACK2 (Fig. 1). Although this approach did not allow the simultaneous identification of ICC and macrophages, it permitted us to relate populations identified by FCM with those observed microscopically.

The total cell counts in the suspensions obtained from individual 9- to 13-day old small intestines (jejunum + ileum) varied between $0.86 \times 10^6$ and $1.12 \times 10^6$ (mean = $1.01 \times 10^6$; $n = 3$). The frequencies of ICC and macrophages were $3.26 \pm 0.27$ and $1.67 \pm 0.28\%$, respectively (Fig. 3, A, D, and G), reflecting mean total cell counts of $32.8 \times 10^3$ (ICC) and $16.8 \times 10^3$ (macrophages). MACS (Fig. 3, B, E, and H) resulted in a significant increase in the proportion of the AF 488-ACK2-positive cells (to $28.8 \pm 9.19\%$) relative to both the unsorted and the MACS− groups ($P < 0.009$; Fig. 3, G–I), whereas the proportion of macrophages was marginally reduced (to $1.00 \pm 0.27\%$; not significant) despite their quantitative uptake of the ACK2. It is likely that the latter was due to the largely intracellular localization of the primary antibody.

Fig. 3. Quantitative assessment by FCM of immunomagnetic enrichment of ICC from freshly dispersed small intestinal muscles. A–C: identification by FCM of cells labeled with AF 488-ACK2 (ICC and macrophages are shown within the marked regions) in the unsorted, MACS+, and MACS− fractions. MACS was performed on cells labeled with fluorescent ACK2; dead cells were excluded on the basis of their uptake of PI (only the viable cells are shown). Note enrichment of positive cells in the MACS+ fraction. D–F: identification by FCM of macrophages labeled with FITC-dextran in parallel samples from the same experiment. MACS was performed on cells labeled with nonfluorescent ACK2; only live (PI−) cells were included in the analysis. Note the slight reduction of labeled cells in the MACS+ fraction. G–I: proportions of labeled cells in 3 independent experiments. Each column triplet reflects cell numbers in the fraction identified in A–F. Shaded bars, cells labeled with AF 488-ACK2 (ICC + macrophages); open bars, FITC-dextran-positive cells (macrophages); closed bars, difference between the proportions of AF 488-ACK2-positive and FITC-dextran-positive cells (ICC). Note enrichment of ICC in the MACS+ fractions.
(ACK2) in these cells, which probably prevented the binding of the F(ab')2 secondary antibody labeled with magnetic beads. Subtraction analysis indicated an 8.23 ± 2.09-fold enrichment of ICC to a final frequency of 27.88 ± 9.37% (P < 0.008 relative to unsorted and MACS− cells; Fig. 3, G–I). The calculated number of harvested ICC was (7.1 ± 1.2) × 10³, representing a ~22% rate of recovery. Although the final ICC frequency fell within the range reported, for example, for the Kit-based enrichment of spermatogonia from hamster, mouse, and marmoset testes using the same technique (35), it was clearly too low, for example, for molecular studies, which require highly purified cells. When we cultured the MACS+ cells to evaluate their utility for functional studies, we found that ICC networks occurred more frequently than in primary cultures (not shown). However, the difference did not appear to be great enough to justify the routine use of MACS for direct, one-step purification of ICC. On the other hand, MACS may be a useful technique for preenriching freshly dispersed ICC for fluorescence-activated cell sorting.

The relatively low final ICC frequency was probably due to the nonspecific binding of unlabeled cells to the columns. We estimated that because of the low abundance of ICC in the unsorted suspensions, nonspecific binding of only ~1.8% of the cells applied to the MACS columns would be sufficient to dilute MACS+ ICC to the observed 27.88% mean final concentration. We verified this nonspecific binding in separate experiments by sorting in the absence of the primary or secondary antibodies (not shown). The nonspecific binding occurred despite the use of F(ab')2 secondary antibodies and Ca²⁺-free buffer containing 2% BSA. It may have been facilitated by the elongated shape of the dispersed smooth muscle cells (the most abundant Kit-negative cells), which is maintained even after tissue dissociation. Therefore, we repeated the above experiments using primary cell cultures, where cell detachment with trypsin-EDTA makes even these cells round or oval shaped. We used confluent cultures because Kit immunoreactivity of ICC is stronger in the presence of other cell types (compare Fig. 2, D and E). Each group consisted of cultures derived from the jejunal and ileal muscles of a single 9- to 13-day-old BALB/c mouse.

Total cell counts in the suspensions obtained from the primary cultures were about fourfold higher than in suspensions freshly dispersed from the same amount of tissue (range = 2.65–6.08 × 10⁶; mean = 4.28 × 10⁶; n = 3). The mean number of macrophages also increased about fourfold (to 64.6 × 10³, representing 0.45 ± 0.10% of the total count), suggesting that on average the cells in primary cultures underwent about two doublings. However, the frequency of ICC was only 0.22 ± 0.15% (Fig. 4, A, D, and G), reflecting a total number of 31.0 × 10³, which was less than the number of ICC in freshly dispersed tissues. In view of the well-known reduction of Kit expression by ICC in culture (see, e.g., Fig. 2, A, D, and E), which occurs without a loss of cells with ICC morphology (31), these findings probably reflect a reduction in the number of ICC identifiable with immunostaining and FCM rather than a true reduction in their total number. In fact, assuming that ICC underwent the same number of doublings as other cells in the cultures (which is supported by the preservation of morphologically identifiable ICC networks in these cultures), it appears that only one in every four ICC remained detectable by FCM, even though the remaining Kit-positive ICC appeared to have relatively high levels of fluorescence (Fig. 4A). It is possible that only the ICC that were in physical contact with cells expressing the membrane-bound isoform of SCF, the physiologically relevant ligand for Kit, continued to express Kit at detectable levels (31) (see also Fig. 2E). MACS (Fig. 4, B, E, and H) increased the proportion of both AF 488-ACK2-positive cells (to 19.06 ± 3.58%; P < 0.001; Fig. 4, G–I) and macrophages (to 2.37 ± 0.80%; P = 0.026; Fig. 4, G–I). Similar to the suspensions derived from freshly dispersed muscles, the lower enrichment of macrophages was probably due to the largely intracellular localization of the ACK2 in these cells. Subtraction analysis indicated 63.28 ± 20.41-fold enrichment of ICC to a final frequency of 16.69 ± 2.81% (P < 0.001; Fig. 4, G–I). The calculated number of harvested ICC was (14.1 ± 3.2) × 10³, indicating a ~46% apparent rate of recovery. ICC detectable by FCM were practically depleted from the MACS− fraction (Fig. 4, C, F, and I). These results are very similar to those reported for immunomagnetic sorting of other rare cell types (22). Thus MACS appeared to be an effective technique to sort ICC from cultured cells, and the relatively low final ICC frequency may not have reflected the true proportion of these cells. The main difference between FCM and MACS is that in the latter the primary immunoreaction is amplified by the paramagnetic secondary antibodies, whereas detection by FCM depends entirely on the abundance of the bound primary antibodies. Therefore, ICC that had too few AF 488-ACK2 antibody molecules attached could have escaped detection by FCM while being retained on the column by the higher number of secondary antibody molecules labeled with the magnetic beads. It is also possible that cultured ICC with low levels of Kit expression were retained on the columns by nonspecific mechanisms. To verify that the efficacy of the magnetic sorting was higher than suggested by FCM, we recultured both the MACS+ and MACS− cells (see below). We did not attempt to use these cells in molecular studies because ICC gene expression is known to undergo changes in culture (7).

In primary cultures grown beyond confluence, ICC typically proliferate and form extensive networks on the surface of a cell layer mainly composed of smooth muscle cells (Fig. 5A). As predicted by FCM, secondary cultures of MACS− cells contained few ICC and were dominated by smooth muscle cells (Fig. 5B). When we recultured the MACS+ fractions (n = 24), despite the relatively low final ICC concentration suggested by FCM, we frequently observed extensive, nearly homogenous ICC networks that populated large areas of the culture dishes (Fig. 5C). Although these networks were dominated by multipolar (i.e., pacemaker-type) ICC, elongated (possibly intramuscular) ICC were also recognizable. In some cases, ICC networks appeared to be mixed with few smooth muscle cells and flat, large, fibrocyte-like cells (a similar cell is marked with an arrow in Fig. 5C). These findings imply that the MACS+ fractions contained more ICC than suggested by FCM analysis based on Kit immunofluorescence. To obtain further proof, we examined Kit-like immunoreactivity in such secondary cultures (n = 15). Indeed, in cultures with pure ICC networks, Kit immunostaining was weak (Fig. 5D) or barely detectable. In cultures containing cells other than ICC in somewhat greater numbers, Kit-like immunoreactivity was clearly recognizable. Reduction of Kit expression in cultured ICC (see also Ref. 31) is probably a sign of their dedifferentiation. ICC in culture tend
to lose their typical phenotype with time and express smooth muscle myosin mRNA (7) and desmin-like immunoreactivity (T. Ördög, unpublished observations). A change toward a smooth muscle phenotype is also supported by the observation that these cells develop contractile activity (36). Therefore, an alternative explanation for the unexpected homogeneity of the ICC networks in secondary cultures is that the dedifferentiating ICC could have preferentially survived sorting and passaging and/or may have “outgrown” other cell types. Although the exact mechanism of the MACS technique’s selectivity remains obscure, these observations suggest that it is an efficient and sensitive method of purifying ICC from primary cultures, where nonspecific binding of contaminating cells to the columns is lower than in the case of suspensions obtained by collagenase digestion.

However, the reduced Kit-like immunoreactivity in secondary ICC cultures raised the possibility that the cells might have lost their functional characteristics. Therefore, we examined whether networks of ICC derived from MACS+ cells displayed rhythmic pacemaker activity. We could not investigate functions specific for ICC that mediate neuromuscular neurotransmission because at present that function is only recognizable in intact tissues (7, 10).

We used rhod-2 imaging to detect oscillations in mitochondrial \([Ca^{2+}]\), a critical step in the sequence of intracellular events that lead to electrical pacemaking (24, 33, 44). In each culture examined \((n = 10)\), we detected rhythmic oscillations in rhod-2 fluorescence (Fig. 5E) that occurred at a frequency of 13.65 ± 0.73 cycles per minute. This frequency is very similar to the frequencies of rhythmic oscillations recorded previously in cultured murine small intestinal ICC at similar temperatures by various techniques, including patch-clamp recordings of transmembrane currents or voltage (16, 18, 36, 44) and imaging of mitochondrial (44) or cytoplasmic \([Ca^{2+}]\) concentrations (T. Ördög and K. M. Sanders, unpublished observations). These findings indicate that purified and recultured ICC are capable of generating a normal pacemaker rhythm.

Although the intracellular mechanism for activation of pacemaker activity appeared to be intact, it is possible that secondary cultures of ICC might lose the ionic apparatus necessary to generate slow waves. Thus we also performed experiments with TMRM, a positively charged potentiometric dye that can undergo potential-dependent redistribution across cellular and intracellular membranes (8). Since mitochondrial membrane potential is more negative than the potential in the cytoplasm, TMRM accumulates in these organelles and displays a further
increase in its fluorescence on depolarization of the cell membrane (8) (Fig. 5F). We verified the latter by depolarizing cultured ICC with elevated external K⁺ (not shown). In each culture studied (n = 14), rhythmic oscillations in membrane potential, as reported by TMRM, were observed (Fig. 5G). These oscillations occurred at a frequency of 13.68 ± 0.22 cycles per minute, indicating that the electrical pacemaker apparatus in these cells remained intact. These findings indicate that ICC networks obtained by immunomagnetic purification of cultured ICC are capable of generating rhythmic electrical pacemaker activity and underlying oscillations in mitochondrial Ca²⁺ concentration. The parameters of the rhythmic activity are indistinguishable from those detected in mixed primary cultures (16, 18, 24, 29, 36–38, 44). Others have recorded from ICC that grew out of small tissue explants (14) or from ICC identified in intact tissues (6, 30, 46). Our approach may enable studies that have not been possible with currently available techniques, such as the effects of network size on slow-wave amplitude (16, 29), the role of network expansion in the normal development and maturation of the GI pacemaker apparatus (39, 43), the mechanisms of pacemaker integration in ICC networks (15, 24, 30, 46), the mechanisms of arrhythmias caused by focal damages in the ICC networks (24, 26, 1), and the modulatory effects of electrically coupled smooth muscle cells on electrical pacemaking.

In this study, we demonstrate that immunomagnetic sorting of ICC from primary cultures of murine GI muscles can yield sufficiently pure, functionally intact ICC networks. Normal electrical pacemaker activity is preserved in the sorted and recultured ICC despite a significant reduction in their Kit expression. The purified ICC networks appear to be particularly suitable for the in-depth analysis of the mechanisms of
pacemaker integration. However, this technique can only moderately enrich, but not purify, ICC from freshly dispersed cell suspensions and therefore is not practical for obtaining ICC for molecular studies that require high purity. FCM can be used for quantitative analysis of ICC, although low Kit expression may limit its utility in cultured cells, at least if it is used in connection with direct immunofluorescent labeling. Accurate assessment of the number and proportion of ICC in normal or diseased tissues by FCM will require the simultaneous identification of resident macrophages.

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