JCMM
Available online at
www.jcmm.ro
www.jcmm.org

J. Cell. Mol. Med. Vol 9, No 2, 2005 pp. 407-420

C-kit immunopositive interstitial cells (Cajal-type) in human myometrium

Sanda M. Ciontea [a], E. Radu [a,c], T. Regalia [a], Laura Ceafalan [a], D. Cretoiu [a,c], Mihaela Gherghiceanu [c], R.I. Braga [b], Mariana Malincenco [a], L. Zagrean [b], M.E. Hinescu [a,c], L.M. Popescu [a,c] *

[a] Department of Cellular and Molecular Medicine,
[b] Department of Physiology,
"Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
[c] "Victor Babes" National Institute of Pathology, Bucharest, Romania

Received: April 28, 2005; Accepted: May 30, 2005

Abstract

Previous reports describing Cajal-like interstitial cells in human uterus are contradictory in terms of c-kit immunoreactivity: either negative (but vimentin-positive) in pregnant myometrium, or positive, presumably in the endometrium. The aim of this study was to verify the existence of human **m**yometrial **C**ajal-**l**ike **i**nterstitial **c**ells (m-CLIC). Six different, complementary approaches were used: 1) *methylene-blue supravital staining* of tissue samples (cryosections), 2) methylene blue and *Janus green B vital staining* (m-CLIC and mitochondrial markers, respectively), and 3) extracellular *single-unit* electrophysiological *recordings in cell cultures*, 4) *non-conventional light microscopy* on glutaraldehyde/osmium fixed, Epon-embedded semi-thin sections (less than 1μm) stained with toluidine blue (TSM), 5) *transmission electron microscopy* (TEM), and 6) *immunofluorescence* (IF). We found m-CLIC in myometrial cryosections and in cell cultures. *In vitro*, m-CLIC represented ~7% of the total cell number. m-CLIC had 2-3 characteristic processes which were very long (~60μm), very thin (≤0.5μm) and moniliform. The dilated portions of processes usually accomodated mitochondria. *In vitro*, m-CLIC exhibited spontaneous electrical activity (62.4±7.22 mV field potentials, short duration: 1.197±0.04ms). Moreover, m-CLIC fulfilled the usual TEM criteria, the so-called 'gold' or 'platinum' standards (*e.g.* the presence of discontinuos basal lamina, caveolae, endoplasmic reticulum, and close contacts between each other, with myocytes, nerve fibers and/or capillaries etc.). IF showed that m-CLIC express CD117/c-kit, sometimes associated with CD34 and with vimentin along their processes.

In conclusion, we describe myometrial Cajal-like interstitial cells that have affinity for methylene blue and Janus green B vital dyes, fulfill (all) TEM criteria, express CD117/c-kit and have spontaneous electric activity.

Keywords: CD117/c-kit • CD34 • immunofluorescence • human myometrium • smooth muscle α-actin • vimentin • methylene-blue vital staining • caveolae • interstitial cells • spontaneous electrical activity

Introduction

During the last 20 years convincing data accumulated showing that the interstitial cells of Cajal (ICC) are located along the digestive tube [1–3]. Such cells have an essential role as pacemakers and possible regulators of neurotransmission [4–6]. More recently, Cajal-like interstitial cells have been described (based on c-kit immunopositivity and/or TEM) outside the gastrointestinal tract: ureter [7, 8], bladder [9–11], urethra [12], vas deferens [13], fallopian tube [14], blood vessels [15, 16], lymphatics [17], pancreas [18], prostate [19, 20], mammary gland [21]. Conflicting

* Correspondence to: L.M. POPESCU, M.D., Ph.D.
Department of Cellular and Molecular Medicine,
"Carol Davila" University of Medicine and Pharmacy,
P.O. Box 35-29, Bucharest 35, Romania.
E-mail: LMP@univermed-cdgm.ro
LMP@jcmm.org

Reprinted from:
Journal of Cellular and Molecular Medicine

Published by:
the CMM Foundation

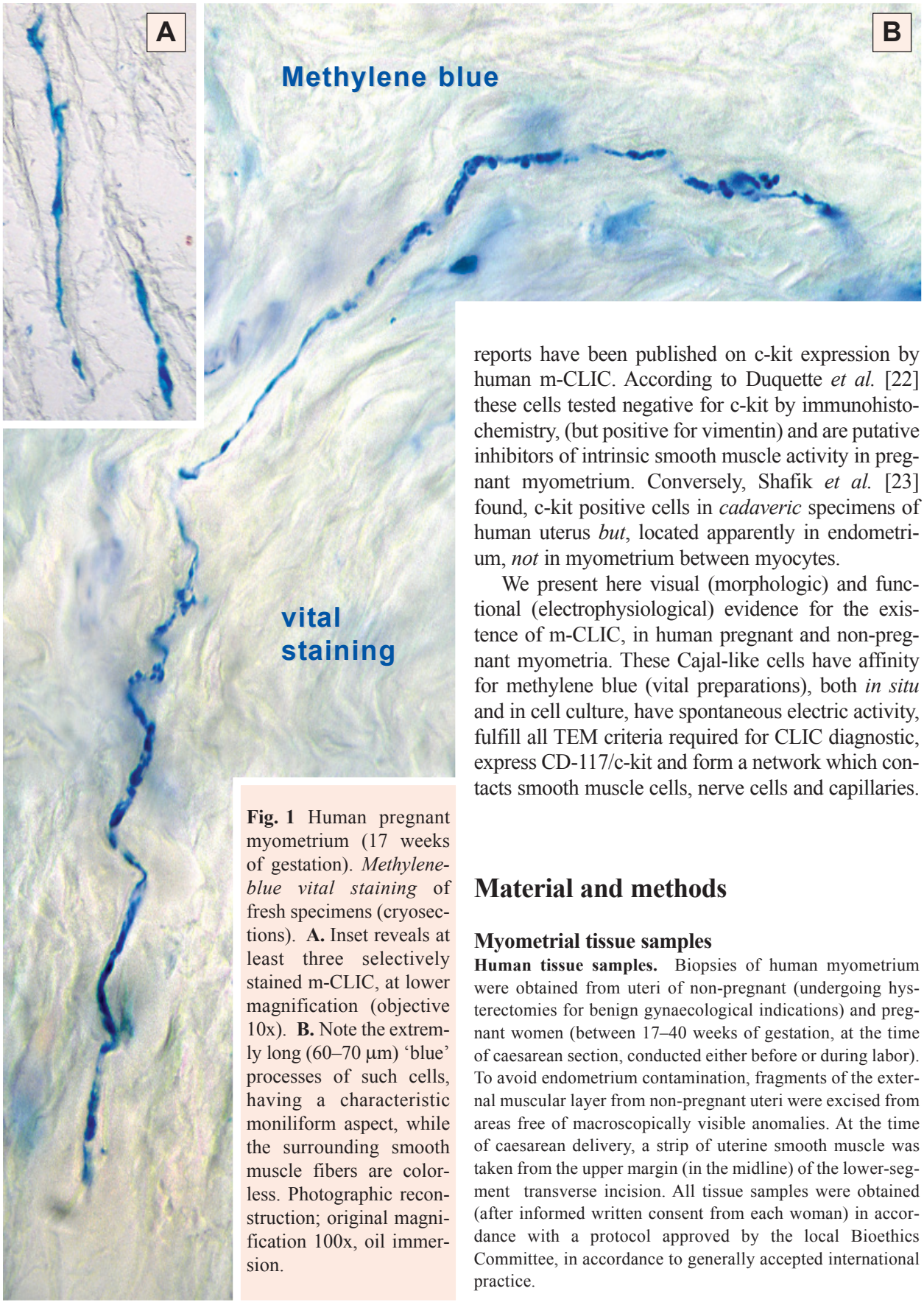


Fig. 1 Human pregnant myometrium (17 weeks of gestation). *Methylene-blue vital staining* of fresh specimens (cryosections). **A.** Inset reveals at least three selectively stained m-CLIC, at lower magnification (objective 10x). **B.** Note the extremly long (60–70 µm) 'blue' processes of such cells, having a characteristic moniliform aspect, while the surrounding smooth muscle fibers are colorless. Photographic reconstruction; original magnification 100x, oil immersion.

reports have been published on c-kit expression by human m-CLIC. According to Duquette *et al.* [22] these cells tested negative for c-kit by immunohistochemistry, (but positive for vimentin) and are putative inhibitors of intrinsic smooth muscle activity in pregnant myometrium. Conversely, Shafik *et al.* [23] found, c-kit positive cells in *cadaveric* specimens of human uterus *but*, located apparently in endometrium, *not* in myometrium between myocytes.

We present here visual (morphologic) and functional (electrophysiological) evidence for the existence of m-CLIC, in human pregnant and non-pregnant myometria. These Cajal-like cells have affinity for methylene blue (vital preparations), both *in situ* and in cell culture, have spontaneous electric activity, fulfill all TEM criteria required for CLIC diagnostic, express CD-117/c-kit and form a network which contacts smooth muscle cells, nerve cells and capillaries.

Material and methods

Myometrial tissue samples

Human tissue samples. Biopsies of human myometrium were obtained from uteri of non-pregnant (undergoing hysterectomies for benign gynaecological indications) and pregnant women (between 17–40 weeks of gestation, at the time of caesarean section, conducted either before or during labor). To avoid endometrium contamination, fragments of the external muscular layer from non-pregnant uteri were excised from areas free of macroscopically visible anomalies. At the time of caesarean delivery, a strip of uterine smooth muscle was taken from the upper margin (in the midline) of the lower-segment transverse incision. All tissue samples were obtained (after informed written consent from each woman) in accordance with a protocol approved by the local Bioethics Committee, in accordance to generally accepted international practice.

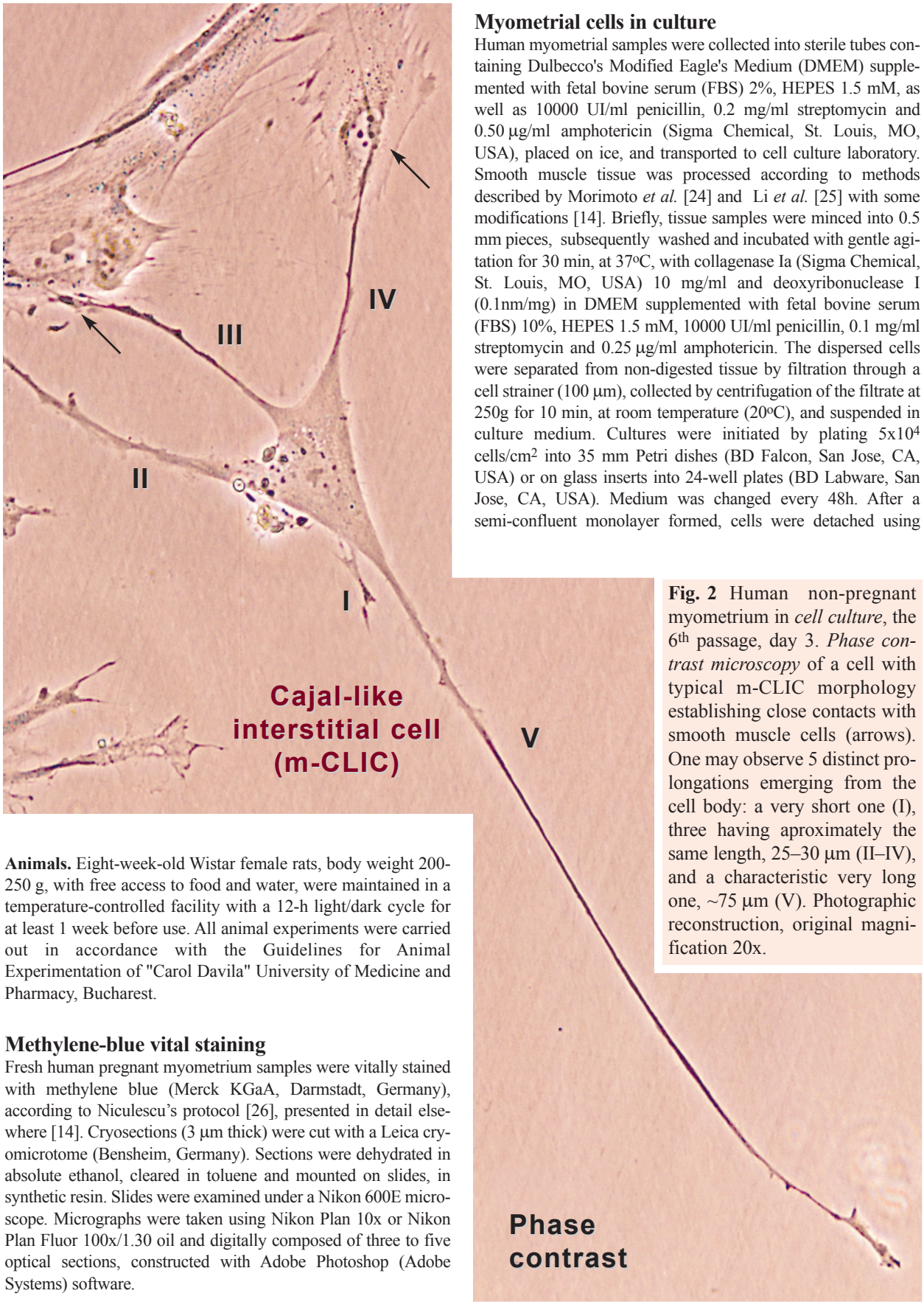


Fig. 2 Human non-pregnant myometrium in *cell culture*, the 6th passage, day 3. *Phase contrast microscopy* of a cell with typical m-CLIC morphology establishing close contacts with smooth muscle cells (arrows). One may observe 5 distinct prolongations emerging from the cell body: a very short one (I), three having aproximately the same length, 25–30 µm (II–IV), and a characteristic very long one, ~75 µm (V). Photographic reconstruction, original magnification 20x.

Myometrial cells in culture

Human myometrial samples were collected into sterile tubes containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (FBS) 2%, HEPES 1.5 mM, as well as 10000 UI/ml penicillin, 0.2 mg/ml streptomycin and 0.50 µg/ml amphotericin (Sigma Chemical, St. Louis, MO, USA), placed on ice, and transported to cell culture laboratory. Smooth muscle tissue was processed according to methods described by Morimoto *et al.* [24] and Li *et al.* [25] with some modifications [14]. Briefly, tissue samples were minced into 0.5 mm pieces, subsequently washed and incubated with gentle agitation for 30 min, at 37°C, with collagenase Ia (Sigma Chemical, St. Louis, MO, USA) 10 mg/ml and deoxyribonuclease I (0.1nm/mg) in DMEM supplemented with fetal bovine serum (FBS) 10%, HEPES 1.5 mM, 10000 UI/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/ml amphotericin. The dispersed cells were separated from non-digested tissue by filtration through a cell strainer (100 µm), collected by centrifugation of the filtrate at 250g for 10 min, at room temperature (20°C), and suspended in culture medium. Cultures were initiated by plating $5x10^4$ cells/cm^2 into 35 mm Petri dishes (BD Falcon, San Jose, CA, USA) or on glass inserts into 24-well plates (BD Labware, San Jose, CA, USA). Medium was changed every 48h. After a semi-confluent monolayer formed, cells were detached using

Animals. Eight-week-old Wistar female rats, body weight 200-250 g, with free access to food and water, were maintained in a temperature-controlled facility with a 12-h light/dark cycle for at least 1 week before use. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of "Carol Davila" University of Medicine and Pharmacy, Bucharest.

Methylene-blue vital staining

Fresh human pregnant myometrium samples were vitally stained with methylene blue (Merck KGaA, Darmstadt, Germany), according to Niculescu's protocol [26], presented in detail elsewhere [14]. Cryosections (3 µm thick) were cut with a Leica cryomicrotome (Bensheim, Germany). Sections were dehydrated in absolute ethanol, cleared in toluene and mounted on slides, in synthetic resin. Slides were examined under a Nikon 600E microscope. Micrographs were taken using Nikon Plan 10x or Nikon Plan Fluor 100x/1.30 oil and digitally composed of three to five optical sections, constructed with Adobe Photoshop (Adobe Systems) software.

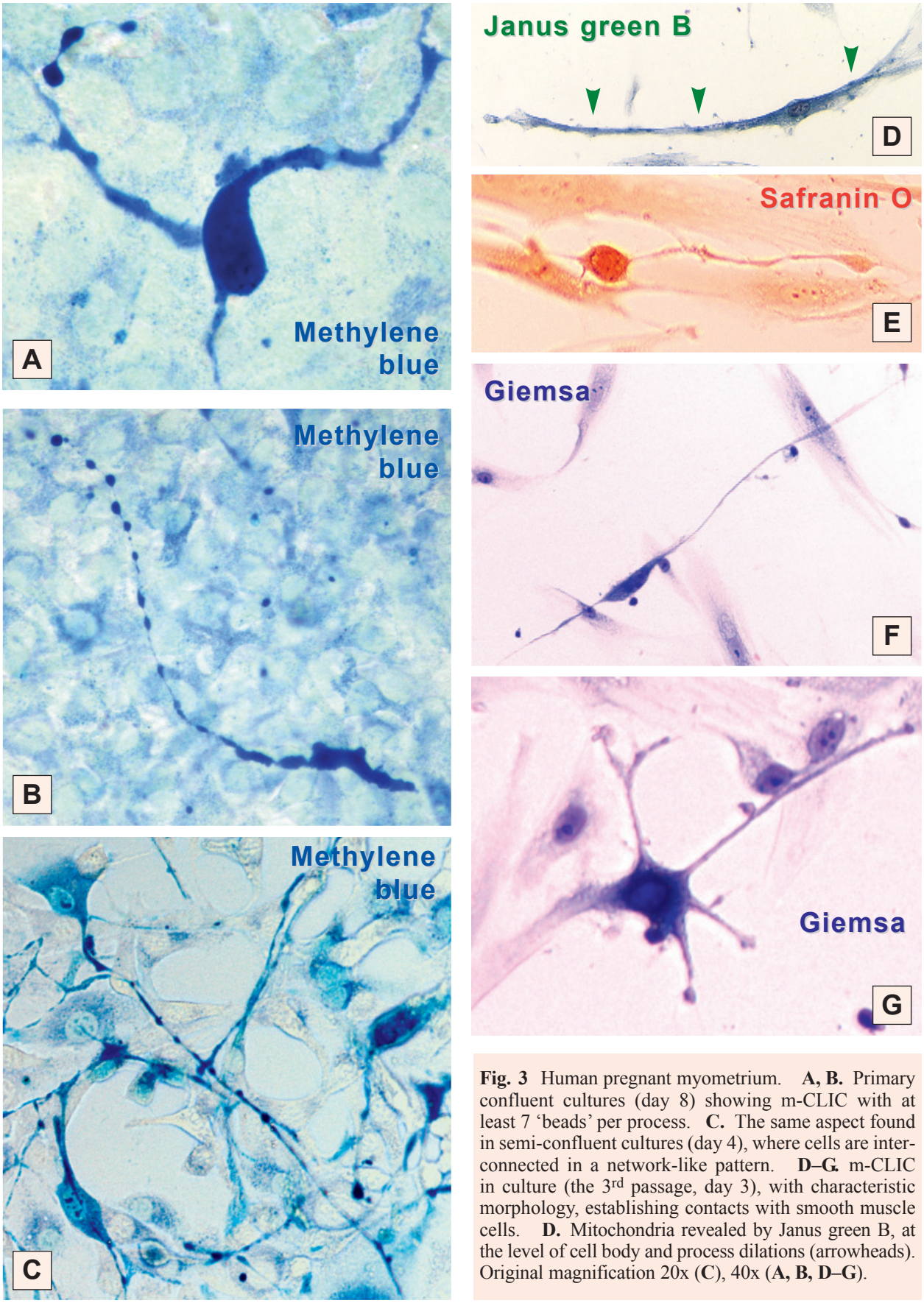


Fig. 3 Human pregnant myometrium. **A, B.** Primary confluent cultures (day 8) showing m-CLIC with at least 7 'beads' per process. **C.** The same aspect found in semi-confluent cultures (day 4), where cells are interconnected in a network-like pattern. **D–G.** m-CLIC in culture (the 3rd passage, day 3), with characteristic morphology, establishing contacts with smooth muscle cells. **D.** Mitochondria revealed by Janus green B, at the level of cell body and process dilations (arrowheads). Original magnification 20x (**C**), 40x (**A, B, D–G**).

0.25% trypsin and 2mM EDTA and replated at a density of $5x10^4$ cells/cm^2, the same technique being used for subsequent passages. Experiments were performed in primary cultures and between passages 1–6.

Methylene blue vital staining. Cells were washed in pre-warmed phenol red-free DMEM (Sigma Chemical, St. Louis, MO, USA), and incubated for 20 min, in a 0.02% methylene-blue solution, at 37°C. Methylene-blue solutions with dilutions between 0.01–0.1% have been tested and 0.02% was chosen as being optimal. Cells were examined and photographed under a Nikon inverted TE200 microscope equipped with a Nikon DN-5 digital camera.

Janus green B vital staining. Cells in culture, the 3rd passage, were stained for 30 min in 0.02% Janus green B (Sigma Chemical, St. Louis, MO, USA) [14] in DMEM and maintained at 37°C, in a humidified atmosphere, 5% CO_2 in air. After repeated washes in DMEM, cells were examined under a heated object-stage inverted Nikon TE200 microscope and photographed.

Giemsa staining. The 35 mm Petri culture-dishes were emptied of the culture medium and cells were stained with 0.4% Giemsa solution (Sigma Chemical, St. Louis, MO, USA) in methanol and distilled water (pH 6.9), for 30 min, at room temperature. This method is not a vital one, since the dye and the fixation agent (methanol) are acting concomitantly on live cells. Cells were washed three times in distilled water and examined.

Safranin O. Myometrial cultured cells, grown on coverslips, were fixed with paraformaldehyde 2%, for 30 min, at 4°C, washed 3 times with phosphate buffer saline (PBS), permeabilised with methanol 70%, for 10 min, at 4°C and washed again with PBS. Coverslips were immersed in 0.2% safranin O solution (Sigma Chemical, St. Louis, MO, USA), in PBS, for 2 h, at room temperature. After a rapid wash cells were examined.

Electrophysiology

Extracellular single-unit recordings were performed on m-CLIC cultures (human pregnant myometrium), at the 3rd passage. A Nikon inverted TE200 microscope equipped with a Nikon DN-5 digital camera and an Eppendorf (Eppendorf AG, Hamburg, Germany) NK I micromanipulator were used to position the microelectrodes. On-site made glass microelectrodes (WPI 1B150F-4) were filled with 3 mol/L potassium acetate and had resistances between 4 and 8 MOhms. The field potential variation was measured using a WPI Duo 773 electrometer (World Precision Instruments, Sarasota, FL, USA), and outputs were displayed on an oscilloscope. Electrical signals were digitized using a Digidata 1322A data processor (Axon Instruments, Union City, CA, USA) and digitally stored. The Clampex 8.2 software was used for data acquisition and analysis.

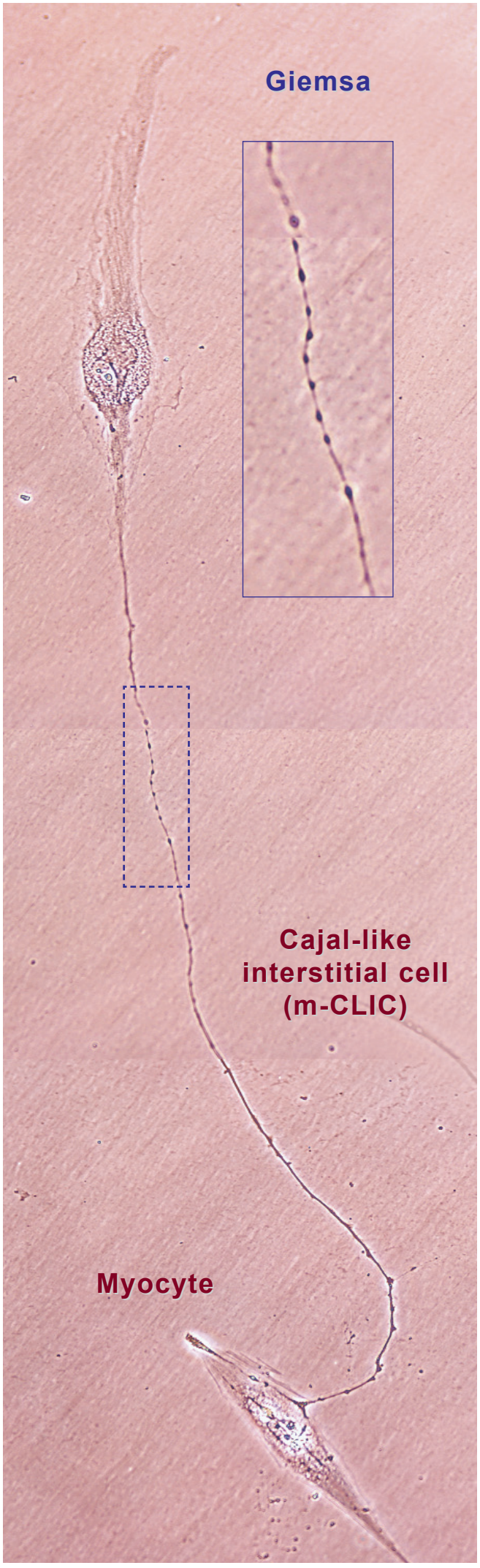
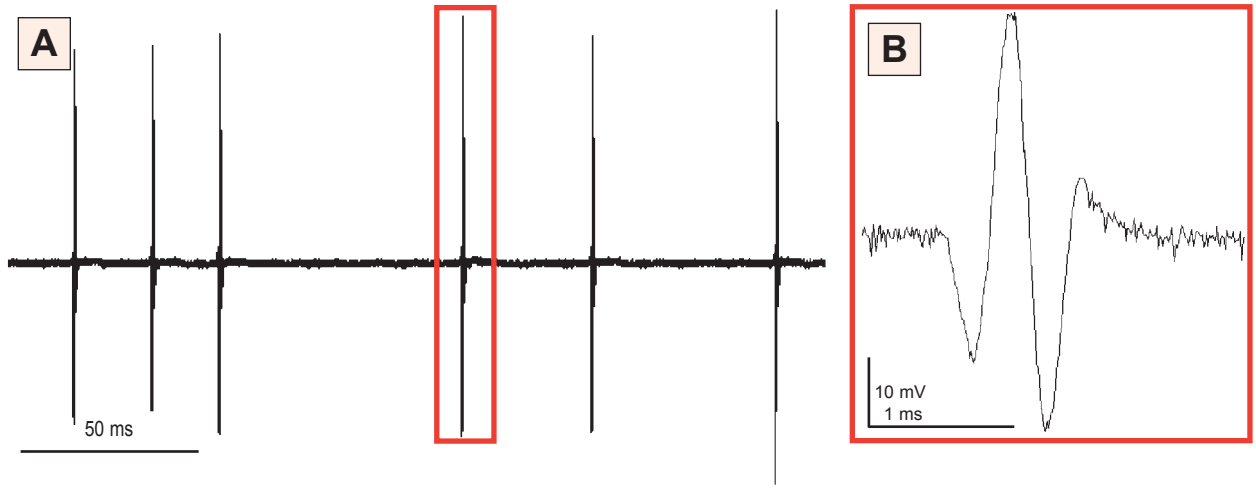


Fig. 4 Human non-pregnant myometrium in cell culture, day 3, the 1st passage. Giemsa staining: pyriform m-CLIC having a close contact with a myocyte. The unique process shows at least 40 specific dilations, 'bead-like', lined up on a ~65 µm 'string'. Photographic reconstruction, original magnification 40x. Inset shows clearly the moniliform aspect at higher magnification.



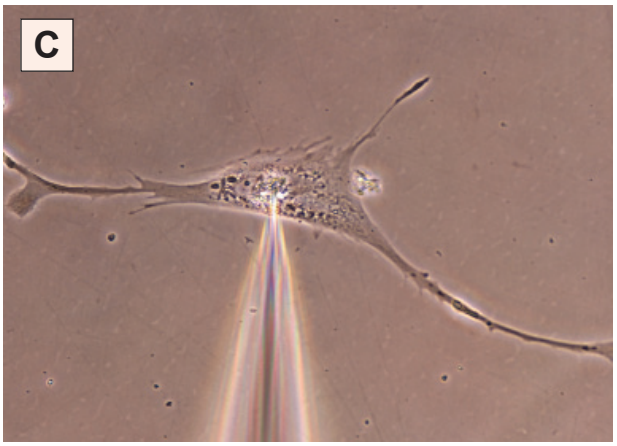


Fig. 5 **A.** Extracellular single-unit recording displaying spontaneous electrical activity in m-CLIC. **B.** Spontaneous field potential pattern. **C.** Phase contrast microscopy (40x) showing the recording glass-electrode at the level of m-CLIC body.

Electron microscopy (TEM)

Electron microscopy was performed on Epon-embedded tissue. Small tissue samples (Wistar rat myometrium), about 1 mm3, were immersed in fresh 4% glutaraldhyde, for 4 h, at 4°C. After fixation, the tissues were washed overnight in 0.1 M sodium cacodylate buffer, at 4°C, post-fixed with 1% potassium ferrocyanide and 1% osmium tetroxide mixture in 0.05 M sodium cacodylate buffer (pH 7.4), at room temperature, for 60 min, dehydrated in graded ethanols and propylene oxide, and embedded in Epon 812. Slides with 1µm thick sections were stained with 1% toluidine blue for optic evaluation. After examination of the toluidine blue-stained sections, ultrathin sections (60 nm) were cut using a MT-7000 ultramicrotome (Research Manufacturing Company Inc., Tucson, AZ, USA), mounted on 50-mesh grids, and double stained with uranyl acetate and lead citrate.The grids were examined in a Phillips 301 electron microscope or in a CM 12 Philips electron microscope, at an acceleration voltage of 60 kV.

Thin-section microscopy (TSM)

Control semi-thin sections (less than 1 µm) were stained with 0.25% Toluidine blue and examined by light microscopy (Nikon Eclipse E600). Representative photomicrographs were taken using Nikon Plan 40x and Nikon Plan Flour 100x/1.30 oil.

Immunofluorescence (IF)

Immunofluorescent staining was performed using a protocol adapted after Mora *et al.* [27]. Cells grown on coverslips were fixed using 2% paraformaldehyde for 10 minutes at room temperature, then washed in PBS and permeabilized in PBS containing 0.5% bovine serumalbumine (BSA) and 0.075% saponine (PBSSA), for 15 min. Primary antibodies (listed in Table 1, together with their respective clones, working dilutions and manufacturers) were applied for 4 h, at room temperature. Polyclonal FITC-labeled goat anti-mouse antibodies (working dilution 1:300, BD Pharmingen, San Jose, CA, USA) were used to detect the primary immune reaction. For double staining experiments, the second reaction was detected using monoclonal rat anti-mouse biotinylated antibodies (1:200, clone A85-1, BD Pharmingen, San Jose, CA, USA) and streptavidin - Alexa Fluor 546 (working dilution 1:200, Molecular Probes, Eugene, OR, USA). Finally, nuclei were counterstained with 1 µg/ml Hoechst 33342 (Sigma Chemical, St.

Table 1 Characteristics of primary antibodies used for immunofluorescence.

Specificity	Clone	Conjugate	Working dilution	Manufacturer
c-kit	Ab81	none	1:100	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Vimentin	V9(3)	none	1:100	Dako, Glostrup, Denmark
CD34	8G12	FITC	1:10	BD Immunocytometry System, San Jose, CA, USA
SMA	1A4	FITC	1:100	Sigma Chemical, St. Louis, MO, USA

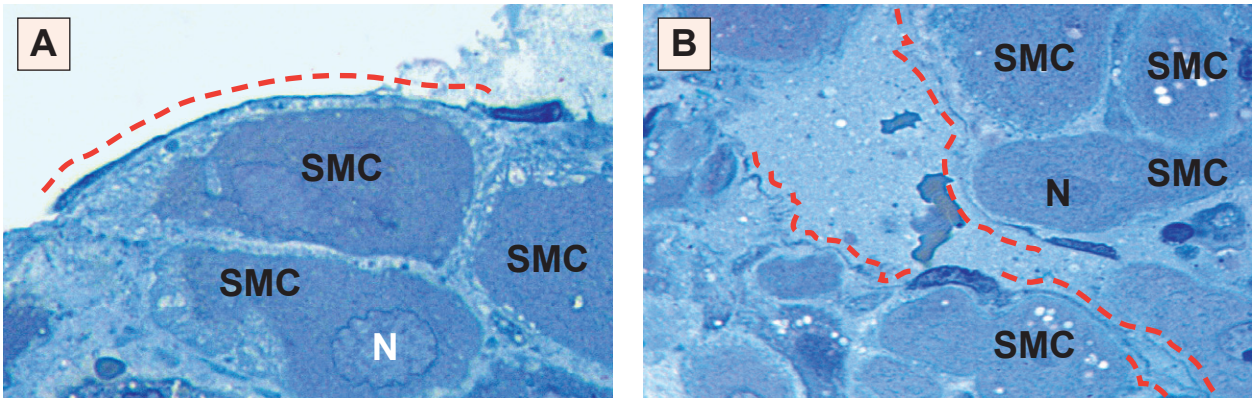


Fig. 6 Human pregnant myometrium (40 weeks of gestation). Non-conventional light microscopy (TSM) on Epon-embedded samples, stained with toluidine blue. Three typical m-CLIC (red dashed lines) are seen embracing the cross-sectioned smooth muscle cells (SMC; N = nucleus). Note very long m-CLIC processes (~ 60 μm). Original magnification 100x, oil immersion.

Louis, MO, USA), and samples examined under a Nikon TE300 microscope equipped with a Nikon DX1 camera, Nikon PlanApo 40x and 60x objectives, and the appropriate fluorescence filters. Negative controls were prepared following the same protocol, but omitting the primary antibodies.

Results

Vital methylene blue staining, the method that led to the initial discovery of Cajal cells in intestine [28], was used to look for Cajal-like interstitial cells in human myometrium. Selectively-stained cells were found in both fresh tissue samples and cell cultures (Figs. 1 & 3A–C). These cells displayed particular morphology, defined by very long, moniliform processes. *In vivo*, m-CLIC prolongations were distributed among the intertwined myometrial fibers, and *in vitro* these cell processes appeared interlaced in a network-like pattern (Fig. 3C). Typical m-CLIC (Fig. 2) were easily recognized in cell cultures (either primary or passages 2–6) by phase contrast microscopy. Quantitatively, m-CLIC represented 6.9±0.9% SEM of myometrial cells grown in primary cultures (n = 651 cells).

Since in a previous study of Cajal-like cells in pancreas [18] we found by TEM that mitochondria are preferentially located in the dilated portions of the cell processes, we used *vital Janus green B* dye,

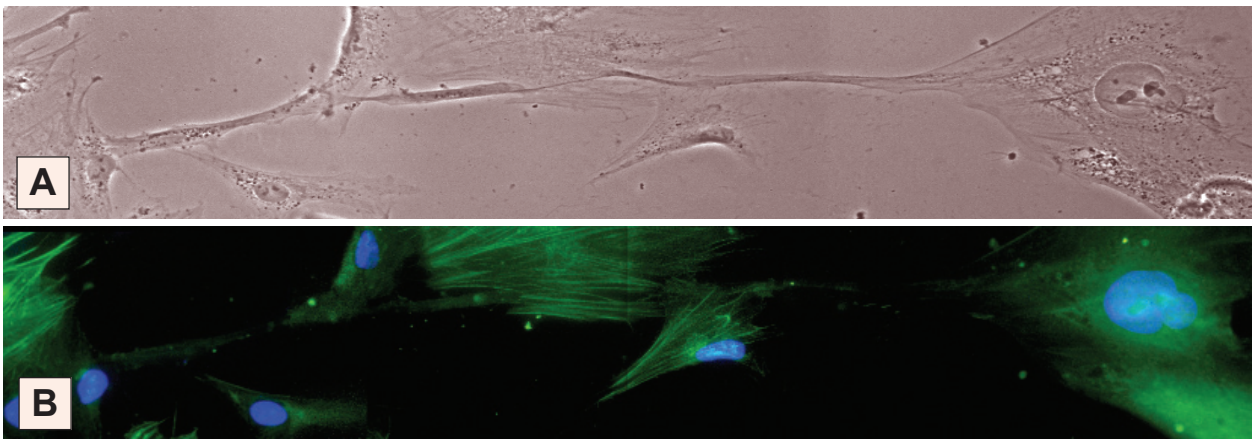


Fig. 7 A, B Human myometrium cell culture (the 2nd passage); control phase-contrast microscopy (**A**) and IF for smooth muscle α-actin (green, **B**) of the same microscopic fields; **B,** typical m-CLIC has one long SMA-negative process that contacts several SMA-positive myocytes. Although the m-CLIC cell body is reactive for SMA, no filamentous pattern is seen. Image reconstruction; original magnification 60x; nuclear counterstaining with Hoechst 33342 (blue).

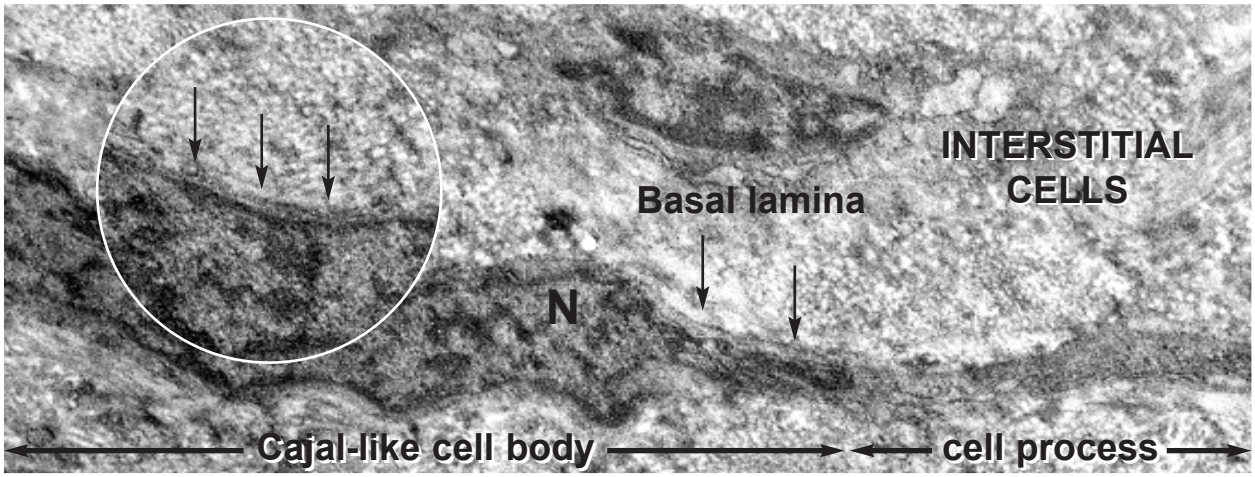


Fig. 8 Human non-pregnant myometrium. TEM; fragment of a Cajal-like interstitial cell (m-CLIC); note the presence of *basal lamina* on the extracellular side of the plasma membrane (arrows). The inset illustrates a higher magnification view of the basal lamina; N = nucleus.

an established mitochondrial marker [29], to show mitochondrial localization in living cells in culture. Indeed, Fig. 3D reveals Janus green B positive 'dots'.

m-CLIC appeared also evident after using less usual dyes. For instance, *safranin O* (Fig. 3E), a dye that stains nuclei in red, actually colored m-CLIC in orange (metachromasia).

Giemsa staining was used since this dye is essentially a mixture of two types of methylene blue dissolved in methanol. Therefore, Giemsa solution acts concomitantly as dye and fixative (Fig. 3F, G). Moreover, our experience suggests that Giemsa staining is the method of choice for revealing the typical moniliform aspect of cell processes of Cajal-like interstitial cells in cultures (Fig. 4).

Extracellular single-unit recordings were done in order to determine whether m-CLIC in cultures exhibit (or not) *spontaneous electrical activity*. Field potentials averaged 62.4 ± 7.22 mV amplitude (n = 30), apparently without a rhythmical pattern (Fig. 5). Compared to the electrophysiological properties of smooth muscle cells [30], the recorded potentials were rather short 1.197 ± 0.04 ms. These results indicate that m-CLIC could generate spontaneous electrical activity.

TSM, the *toluidine-blue staining of semi-thin sections* from human pregnant myometrium depicts m-CLIC in close vicinity of smooth muscle cells. Fig. 6 shows that m-CLIC had an ovoid nucleus surrounded by a relatively small cytoplasm, from which emerged two or three very long (50–75μm) processes.

Although putative m-CLIC can easily be discerned *in vitro* by their long moniliform processes, we tested, by immunofluorescence, the presence of the specific smooth muscle α-actin (SMA) expression. Fig. 7 shows that smooth muscle cells had a characteristic positive reaction for actin stress fibers, while m-CLIC processes appeared negative for SMA (SMA not detectable), but a non-filamentous perinuclear positive immunoreaction is disclosed.

TEM (Figs. 8–10) prove at the ultrastructural level the characteristic features of Cajal-like interstitial cells (the so-called 'gold standard' [4] and /or 'platinum standard' [14, 18]). For instance, Fig. 8 shows clearly the basal lamina. m-CLIC establish, through their processes, vicinity relationships with capillaries (Fig. 9B) and nerve fibers (Fig. 10B), as well as specialized contacts, *gap junctions* with each other and with the smooth muscle cells (Fig. 10 A–D). Noteworthy, we found in myocytes typical '*Ca^{2+} release units*' (caveolae, sarcoplasmic reticulum and mitochondria) [31], vicinal of gap junctions.

Furthermore, to disclose the immunophenotype of m-CLIC, IF assays for c-kit, CD34 and vimentin were used. m-CLIC expressed CD117/c-kit (Fig. 11 A, B) in distinct membrane areas, sometimes associated with CD34 immunoreactivity (Fig. 11C). Cell contacts between m-CLIC processes and surrounding smooth muscle cells were a constant finding, in accordance with the role described for c-kit ligand (expressed on the myocyte) in the development of ICC *in vitro* [32]. Cell processes were also intensely positive for vimentin (Fig. 11D, E), a feature described in previously published reports [33].

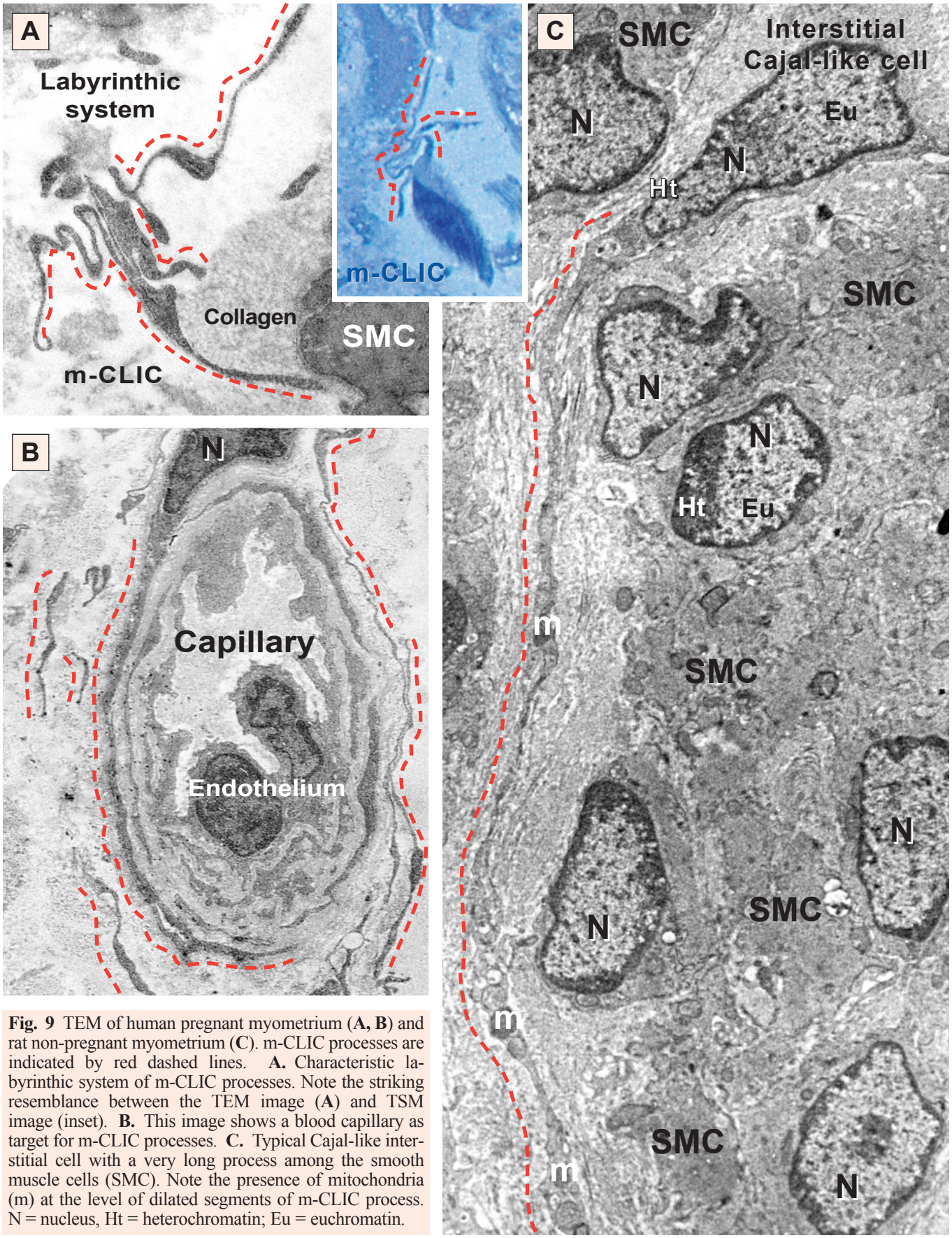


Fig. 9 TEM of human pregnant myometrium (**A, B**) and rat non-pregnant myometrium (**C**). m-CLIC processes are indicated by red dashed lines. **A.** Characteristic labyrinthic system of m-CLIC processes. Note the striking resemblance between the TEM image (**A**) and TSM image (inset). **B.** This image shows a blood capillary as target for m-CLIC processes. **C.** Typical Cajal-like interstitial cell with a very long process among the smooth muscle cells (SMC). Note the presence of mitochondria (m) at the level of dilated segments of m-CLIC process. N = nucleus, Ht = heterochromatin; Eu = euchromatin.

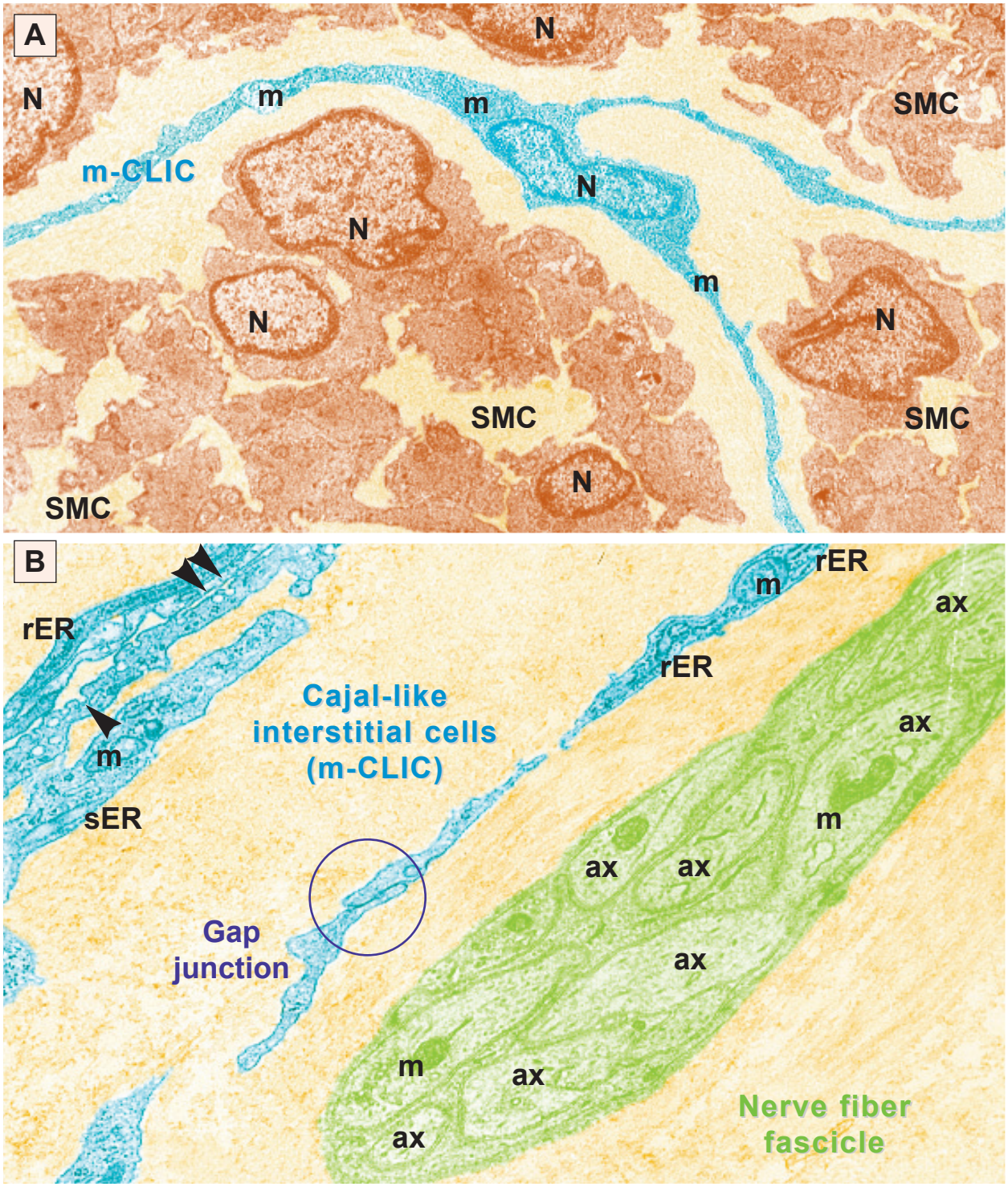
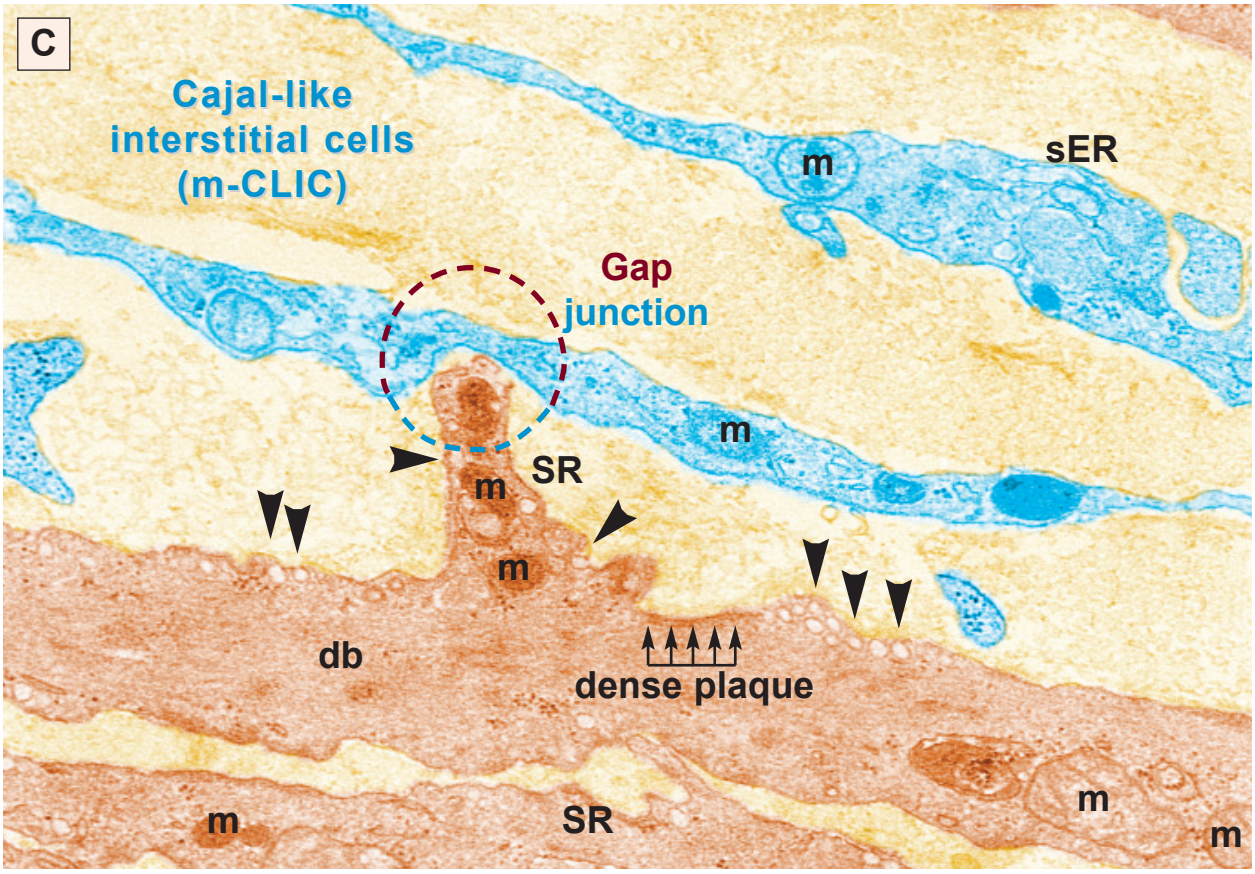
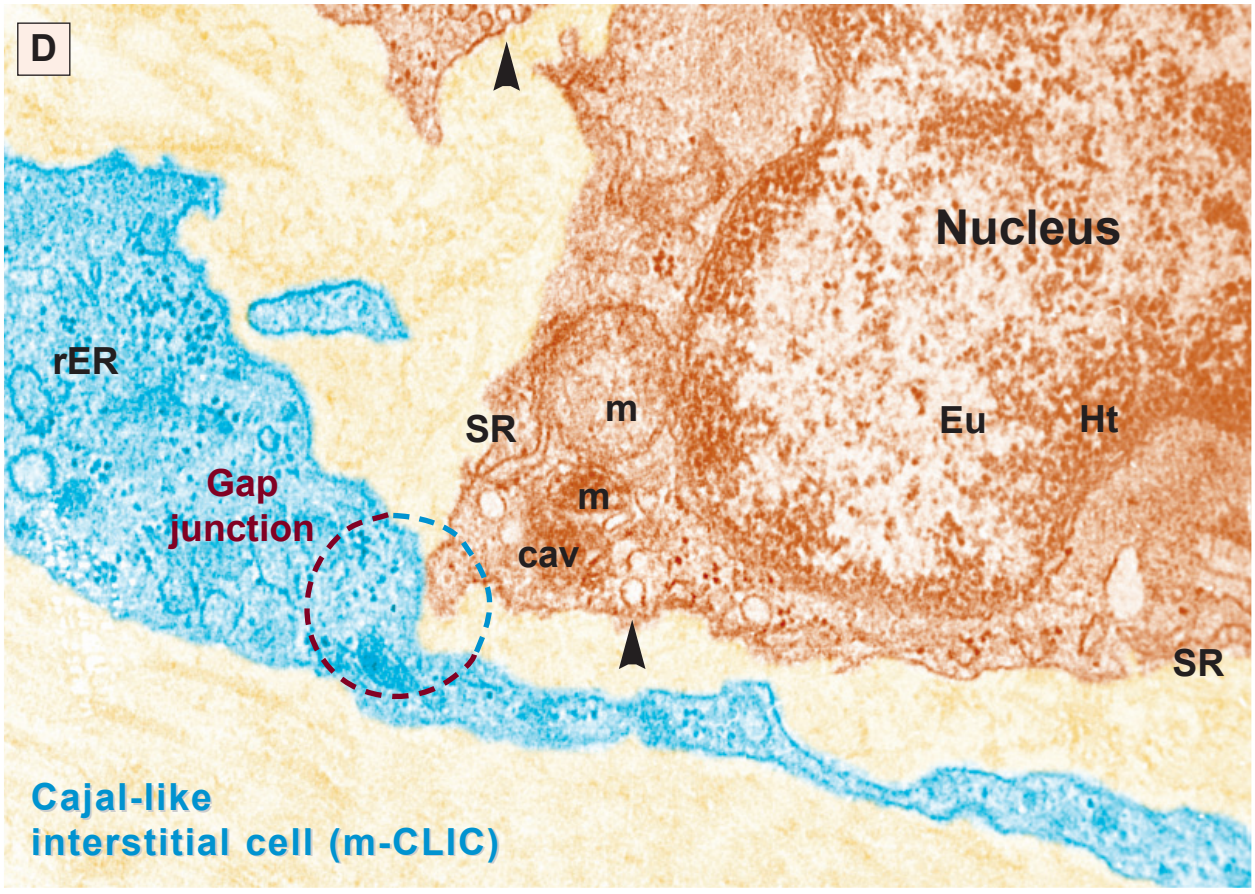


Fig. 10 A–D *Digitally-colored* TEM images of m-CLIC in rat myometrium: Cajal-like interstitial cells (blue), smooth muscle cells (Sienna-brown), and nerve fiber fascicle (green). **A.** Low power micrograph showing *three long, moniliform processes* that encircle bundles of cross-cut smooth muscle cells. Original magnification x6,800. **B.** *Gap junction between two processes of m-CLIC*. The processes are in the proximity of unmyelinated nerve fibers. Original magnification x9,100. **C, D.** *Cell junctions* between myometrial smooth muscle cells and m-CLIC processes. The two membranes are separated by a gap of about 30nm. One may observe '*Ca^{2+}-release units*' (caveolae, sarcoplasmic reticulum and mitochondria) in the cytoplasmic region where smooth muscle sarcolemma comes in close contact with m-CLIC plasmalemma. Original magnification: x11,000 (**C**); x15,000 (**D**). Abbreviations: SMC = smooth muscle cells; ax = axon; db = dense bodies; N = nucleus; Ht = heterochromatin; Eu = euchromatin; rER = rough endoplasmic reticulum; sER = smooth endoplasmic reticulum; SR = sarcoplasmic reticulum; m = mitochondria; cav = caveolae (arrowheads).


C
Cajal-like
interstitial cells
(m-CLIC)
m
sER
Gap
junction
m
SR
m
m
db
dense plaque
m
SR
m
m


D
Nucleus
rER
SR
m
Eu
Ht
Gap
junction
m
cav
SR
Cajal-like
interstitial cell (m-CLIC)

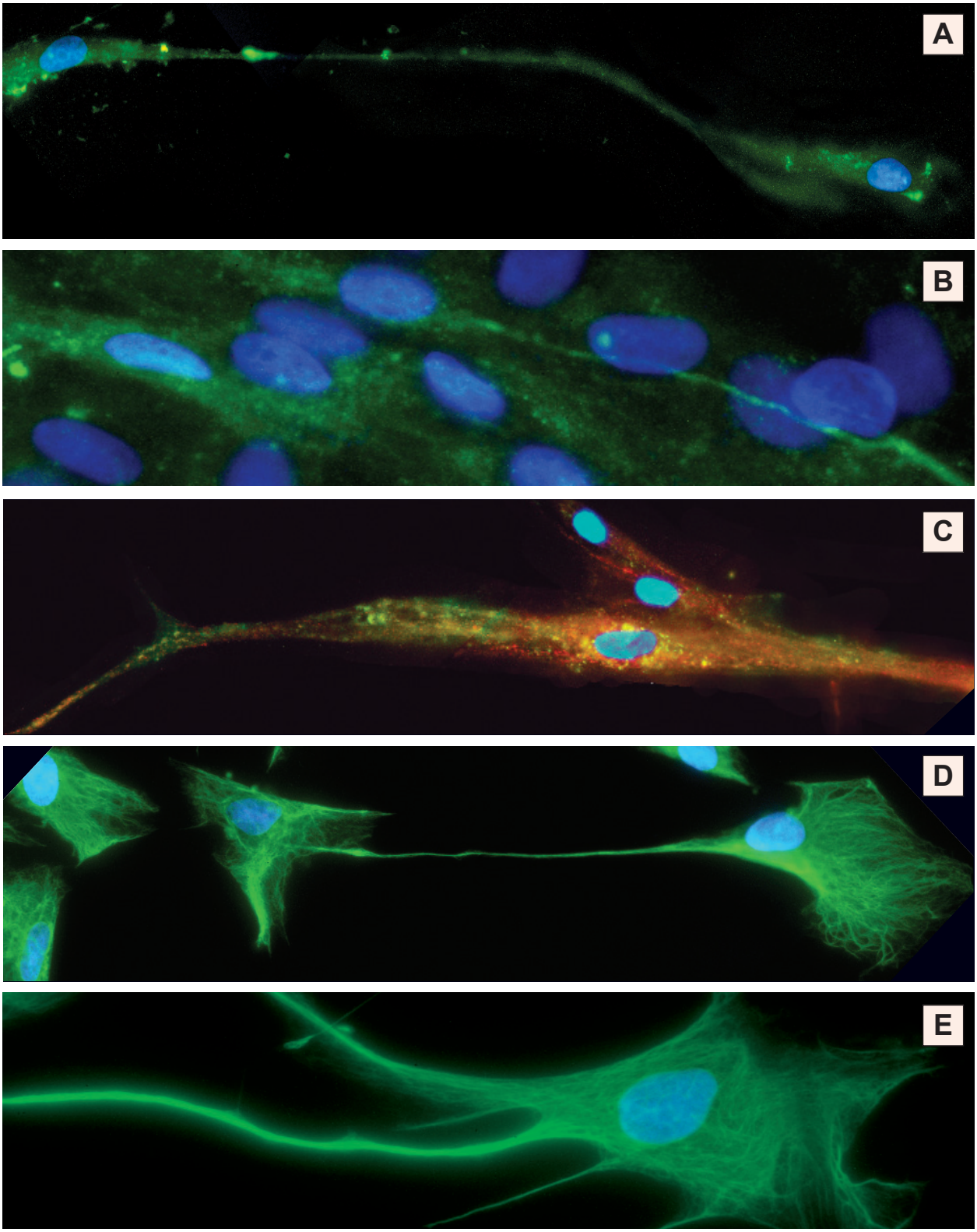


Fig. 11 A–E. Human myometrium cells in culture (the 2nd passage); IF for c-kit (green in A, B), c-kit and CD34 (red and green, respectively in C) and vimentin (green in D, E). Cells that display the m-CLIC morphologic feature (long, moniliform processes) express c-kit and contact adjacent cells (A–C). Some cells suggestive for m-CLIC coexpress c-kit and CD34 (C). The characteristic cell processes are immunoreactive for vimentin and establish connections with nearby cells (D and E). Original magnification 60x, nuclear counterstaining with Hoechst 33342 (blue).

Discussion

This study provides evidence for the presence of a c-kit positive cell type, with Cajal-like morphology, in human pregnant and non-pregnant myometrium. However, a paper reported c-kit non-immunoreactive ICC in human and rat myometrium [22]. These apparently contradictory results could suggest the (co)existence in myometrium of at least two subpopulations of Cajal-like cells, different in terms of c-kit expression, as reported for the human small intestine [34].

In order to deal with the issue of m-CLIC identity, besides c-kit-immunofluorescence, we used 'classical' vital staining with methylene blue and ultrastructural studies (TEM).

Although several sets of criteria have been assambled to identify ICC ('gold standard' [4]) or Cajal-like interstitial cells ('platinum standard' [14, 18]), we emphasize that not all criteria apply to each cell.

In our opinion, any debate on c-kit expression should start after a minimal set of criteria for Cajal-like cells is satisfied. Thus, we looked for Cajal-like interstitial cells, according to the following basic check-list:

- location in non-epithelial (interstitial) spaces;
- vital staining with methylene blue;
- multiple long, thin, moniliform processes.

'Our' m-CLIC fulfill these conditions and, additionaly, have ultrastructural features specific for ICC, different from smooth muscle cells, fibroblasts or myofibroblasts.

Since c-kit IHC is used in almost all current studies on ICC pathology [35], the precise distribution of c-kit expression in normal tissues has to be extensively reconsidered. Examining 3,000 samples from more than 120 different tumor categories, Went *et al.*, provided a list of normal tissues/organs found to be completely c-kit-negative: "cerebrum, uterine cervix, colon, endometrium, esophagus, fat tissue, gall bladder, heart, kidney, liver, lung, lymph node, myometrium, oral cavity mucosa, ovary, pancreas, parathyroid, salivary gland, prostate, skeletal muscle, small intestine, intestinal smooth muscle, stomach mucosa, thyroid, and urothelium" [36]. These data are in contradiction with the 'wave' of papers identifying ICC or ICC-like cells [7-21] in some of the so-called 'completely kit-negative tissues'. Possible explanations might be: a) differences in the affinity of the used antibodies; b) the great sensitivity of ICC to fixation [7].

An indirect argument for the existence of c-kit positive cells in human myometrium might be the occurrence of this antigen in myometrial sarcomas [37]. ICC loss was identified as a cause of bowel motility disorders [38]. It remains to be established if such specific loss of Cajal-like cells could be identified in the pathology of myometrium.

We noted spontaneous electrical activity of m-CLIC in cultures. This m-CLIC behavior probably reflects a closer relationship with typical ICC and a possible role of pace-maker cannot be overlooked.

Direct communication by cellular bridges might be a common morphological feature of stem cells and cancer cells [39, 40]. It is tempting to speculate that our IF data on m-CLIC might represent a similar 'direct' cell to cell communication.

In conclusion, in our opinion, the search for Cajal-like interstitial cells should begin in interstitium, after vital methylene-blue staining revealed cells with characteristic long, moniliform processes. Expression of c-kit and TEM should be the next required steps of the algorythm for Cajal-like interstitial cell diagnostic. Electrophysiology could complete the functional profile of this still enigmatic cell.

References

1. **Thuneberg L,** One hundred years of interstitial cells of Cajal. *Microsc Res Tech*. 1999; 47: 223-38.
2. **Faussone-Pellegrini MS, Pantalone D, Cortesini C.** Smooth muscle cells, interstitial cells of Cajal and myenteric plexus interrelationships in the human colon. *Acta Anat (Basel)*. 1990; 139: 31-44.
3. **Rumessen JJ, Peters S, Thuneberg L.** Light- and electron microscopical studies of interstitial cells of Cajal (ICC) and muscle cells at the submucosal border of human colon, *Lab Invest*. 1993; 68: 481-95.
4. **Huizinga JD, Thuneberg L, Vanderwinden JM, Rumessen JJ.** Interstitial cells of Cajal as targets for pharmacological intervention in gastrointestinal motor disorders. *Trends Pharmacol Sci.* 1997; 18: 393-403.
5. **Ward SM, Sanders KM.** Physiology and pathophysiology of the interstitial cell of Cajal: From Bench to Bedside I. Functional development and plasticity of interstitial cells of Cajal networks. *Am J Physiol Gastrointest Liver Physiol.* 2001; 281: G602-G611.
6. **Hirst GD, Ward SM.** Interstitial cells: involvement in rhythmicity and neural control of gut smooth muscle. *J Physiol.* 2003; 550: 337-46.

7. **Pezzone MA, Watkins SC, Alber SM, King WE, de Groat CW, Chancellor MB, Fraser MO.** Identification of c-kit-positive cells in the mouse ureter: the interstitial cells of Cajal of the urinary tract. *Am J Physiol Renal Physiol.* 2003; 284: 925-9.
8. **Metzger R, Schuster T, Till H, Franke FE, Dietz HG.** Cajal-like cells in the upper urinary tract: comparative study in various species. *Pediatr Surg Int.* 2005; 21: 169-74.
9. **McCloskey KD, Gurney AM.** Kit positive cells in the guinea-pig bladder. *J Urol.* 2002; 168: 832-6.
10. **Blyweert W, Aa F, Ost D, Stagnaro M, Ridder D.** Interstitial cells of the bladder: the missing link? *BJOG*. 2004; 111(s1):57-60.
11. **Davidson RA, McCloskey KD.** Morphology and localization of interstitial cells in the guinea pig bladder: structural relationships with smooth muscle and neurons. *J Urol.* 2005; 173: 1385-90.
12. **Sergeant GP, Hollywood MA, McCloskey KD, Thornbury KD, McHale NG.** Specialized pacemaking cells in the rabbit urethra. *J Physiol.* 2000; 526: 359-66.
13. **Burton LD, Housley GD, Salih SG, Jaeenwood D.** P2X2receptor expression by interstitial cells of Cajal in vas deferens implicated in semen emission. *Auton Neurosci Basic Clin.* 2000; 84: 147-61.
14. **Popescu LM, Ciontea MS, Cretoiu D, Hinescu ME, Radu E, Ionescu N, Ceausu M, Gherghiceanu M, Braga RI, Vasilescu F, Zagrean L, Ardeleanu C.** Novel type of interstitial cell (Cajal-like) in human fallopian tube. *J Cell Mol Med*. 2005; 9: 479-523.
15. **Harhun MI, Gordienko DV, Povstyan OV, Moss RF, Bolton TB.** Function of interstitial cells of Cajal in the rabbit portal vein. *Circ Res.* 2004; 95: 619-26.
16. **Harhun MI, Pucovsky V, Gordienko DV, Povstyan OV, Bolton TB.** Interstitial cells in the vasculature. *J Cell Mol Med*. 2005; 9: 232-43.
17. **McCloskey KD, Hollywood MA, Thornbury KD, Ward SM, McHale NG.** Kit-like immunopositive cells in sheap mesenteric lymphatic vessels. *Cell Tissue Res.* 2002; 310: 77-84
18. **Popescu LM, Hinescu ME, Ionescu N, Ciontea MS, Cretoiu D, Ardeleanu C.** Interstitial cells of Cajal in pancreas, *J Cell Mol Med*. 2005; 9: 169-90.
19. **Exintaris B, Klemm MF, Lang RJ.** Spontaneous slow wave and contractile activity of the guinea pig prostate. *J Urol.* 2002; 168: 315-22.
20. **Van der Aa F, Roskams T, Blyweert W, De Ridder D.** Interstitial cells in the human prostate: a new therapeutic target? *Prostate.* 2003; 56:250-5.
21. **Popescu LM, Andrei F, Hinescu ME.** Snapshots of mammary gland interstitial cells: methylene blue vital staining and c-kit immunopositivity. *J Cell Mol Med.* 2005; 9: 476-7.
22. **Duquette RA, Shmygol A, Vaillant C, Mobasheri A, Pope M, Burdyga T, Wray S.** Vimentin-positive, c-kit-negative interstitial cells in human and rat uterus: a role in pacemaking? *Biol Reprod.* 2005; 72: 276-83.
23. **Shafik A, El-Sibai O, Shafik I.** Identification of c-kit-positive cells in the uterus. *Int J Gynaecol Obstet.* 2004; 87: 254-5.
24. **Morimoto T, Head JR, MacDonald PC, Casey ML.** Thrombospondin-1 expression in human myometrium before and during pregnancy, before and during labor, and in human myometrial cells in culture. *Biol Reprod.* 1998; 59: 862-70.
25. **Li CX, Liu BH, Tong WD, Zhang LY, Jiang YP.** Dissociation, culture and morphologic changes of interstitial cells of Cajal in vitro. *World J Gastroenterol.* 2005; 11: 2838-40.
26. **Niculescu I.** An atlas concerning morphological aspects of visceral nerve endings. 1st ed. Bucharest: Editura Medicala; 1958.
27. **Mora R, Bonilha VL, Marmorstein A, Scherer PE, Brown D, Lisanti MP, Rodriguez-Boulan E.** Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae, and rafts when co-expressed with caveolin-1. *J Biol Chem.* 1999; 274: 25708-17.
28. **Cajal SR.** Les nouvelles idees sur la structure du systeme nerveux chez l'homme et chez les vertebres. 2eme ed. Paris: C. Reinwald & Cie, Libraires-Editeurs, 1895.
29. **Novikoff AB.** Mitochondria in: The Cell ed. By J. Brachet, A. Mirsky, Academic Press Inc., New York, 1961, 299-421.
30. **Farrugia G.** Ionic conductances in gastrointestinal smooth muscles and interstitial cells of Cajal. *Annu Rev Physiol*. 1999; 61: 45-84.
31. **Moore ED, Voigt T, Kobayashi YM, Isenberg G, Fay FS, Gallitelli MF, Franzini-Armstrong C.** Organization of Ca^{2+} release units in excitable smooth muscle of the guinea-pig urinary bladder. *Biophys J.* 2004; 87:1836-47. Erratum in: *Biophys J*. 2004; 87:2914.
32. **Wu JJ, Rothman TP, Gershon MD,** Development of the interstitial cell of cajal: origin, kit dependence and nonneuronal sources of kit ligand. *J Neurosci Res.* 2000; 59: 384-401.
33. **Torihashi S, Ward SM, Nishikawa S, Nishi K, Kobayashi S, Sanders KM,** c-kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. *Cell Tissue Res.* 1995; 280:97.
34. **Horiguchi K, Keef KD, Ward SM.** Distribution of interstitial cells of Cajal in tunica muscularis of the canine rectoanal region. *Am J Physiol Gastrointest Liver Physiol*. 2003; 284: 756-67.
35. **Wang XY, Berezin I, Mikkelsen HB, Der T, Bercik P, Collins SM, Huizinga JD.** Pathology of interstitial cells of Cajal in relation to inflammation revealed by ultrastructure but not immunohistochemistry. *Am J Pathol*. 2002; 160: 1529-40.
36. **Went PT, Dirnhofer S, Bundi M, Mirlacher M, Schraml P, Mangialaio S, Dimitrijevic S, Kononen J, Lugli A, Simon R, Sauter G.** Prevalence of KIT expression in human tumors. *J Clin Oncol*. 2004; 22: 4514-22.
37. **Rushing RS, Shajahan S, Chendil D, Wilder JL, Pulliam J, Lee EY, Ueland FR, van Nagell JR, Ahmed MM, Lele SM.** Uterine sarcomas express KIT protein but lack mutation(s) in exon 11 or 17 of c-KIT. *Gynecol Oncol*. 2003; 91: 9-14.
38. **Sanders KM, Ördög T, Ward SM.** Physiology and pathophysiology of the interstitial cells of Cajal: from bench to bedside: IV. Genetic and animal models of GI motility disorders caused by loss of interstitial cells of Cajal. *Am J Physiol Gastrointest Liver Physiol*. 2002; 282: 747-56.
39. **Rustom A, Saffrich R, Markovic I, Walther P, Gerdes H-H.** Nanotubular highways for intercellular organelle transport. *Science*. 2004; 303: 1007-10.
40. **Vidulescu C, Clejan S, O'Connor KC.** Vesicle traffic through intercellular bridges in DU 145 human prostate cancer cells. *J Cell Mol Med*. 2004; 8: 388-96.