Human placenta:
de visu demonstration of interstitial Cajal-like cells

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Abstract

Traditional interstitial cells of Cajal (ICC) are present in the digestive tube and are supposed to act as pacemakers and neuromodulators. However, interstitial **C**ajal-**l**ike **c**ells (ICLCs) were found outside the gastrointestinal tract, in various organs (*e.g.* ureter, bladder, fallopian tube, uterus, pancreas, mammary gland, myocardium etc.) and looking for such ICLC is a priority in our laboratories. We report here unequivocal visual evidence that ICLCs are present in the mesenchymal tissue of the villi from human term placenta. The following methods were used: **a**. *vital staining* with methylene blue (cryosections); **b**. *silver impregnation* (paraffin sections); **c**. *Epon-embedded sections* (~1µm) of glutaraldehyde/osmium fixed tissue, stained with toluidine blue; **d**. *primary cell cultures* (or second-passage cells) to reveal the characteristic, very long, moniliform cell processes and mitochondrial localization at dilations (molecular fluorescence probe: MitoTracker Green); **e**. *immunofluorescence* for c-kit/CD117 *marker* or other characteristic proteins; **f**. *transmission electron microscopy* to establish the identity of ICLC.
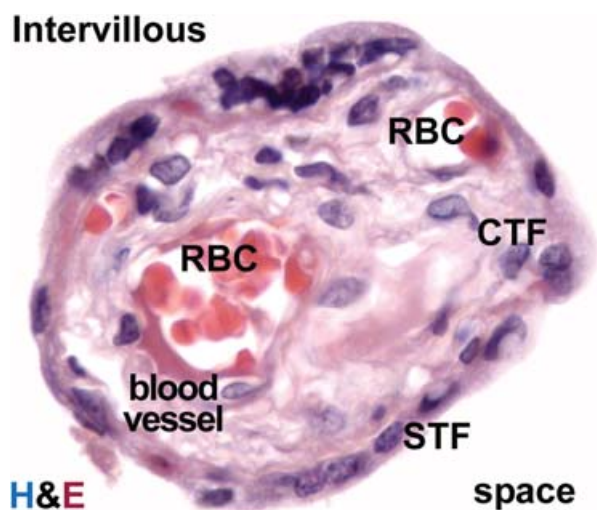
Fig. 1 Human term placenta. Tertiary villus, cross-section, H&E staining, Ob. 100 ×. Note the syncytiotrophoblast (STF) and cytotrophoblast cells (CTF).
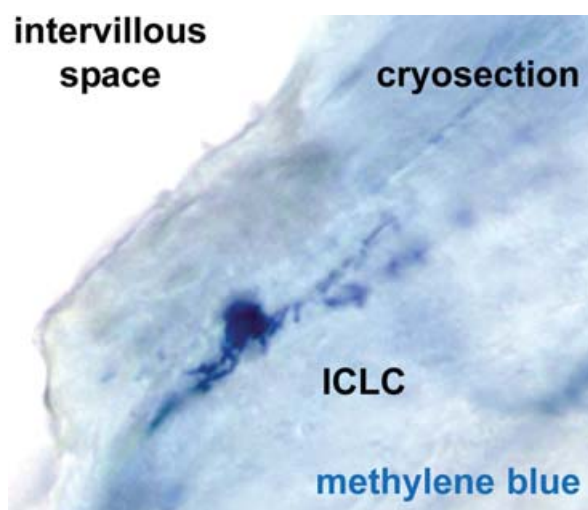
Fig. 2 Human term placenta villus; vital methylene blue staining, cryosection. Note the selective stained ICLC with several (at least 4) moniliform cytoplasmic processes. Ob. 40 ×.

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Fig. 3 Human term placenta villus; oblique section; silver impregnation. Two cells (arrowheads) with long prolongations could be identified in the mesenchymal core of the villus. Ob. 100 ×.

At term, placenta is a thick spherical disk (~20 cm in diameter) with parenchymatous, 'monotonous' microscopic structure. However, the placenta is a remarkable, but *misunderstood* organ [1], which, for the foetus, has at least roles of digestive system, lungs and kidneys. Noteworthy, the placenta is a major endocrine organ, and contrary to (almost) all human organs is not innervated. Composed exclusively of foetal tissues (Fig. 1) placental barrier consists of four layers (mother unto foetus): (a) the external syncytiotrophoblastic layer, (b) the cytotrophoblastic layer, (c) the connective tissue of the villous core – *mesenchymal tissue*, and (d) blood vessel wall.

Demonstration of interstitial **C**ajal-**l**ike **c**ells (ICLCs) [which are similar cells to classical enteric interstitial cells of Cajal (ICC)] in various organs outside the gut became a hot subject in the last 2–3 years [2, 3]. For instance, we have shown the ICLC presence in: human uterus and fallopian tube [3–6], human resting mammary gland [7] or pancreas [8]. ICLCs were also found in human atrial [9] and ventricular myocardium [10].

The aim of this preliminary microscopical investigation was therefore to examine whether ICLCs are present in the interstitial (mesenchymal) villous core of human term placenta.

Human term placentae of uncomplicated pregnancies were obtained by elective Caesarean sections.
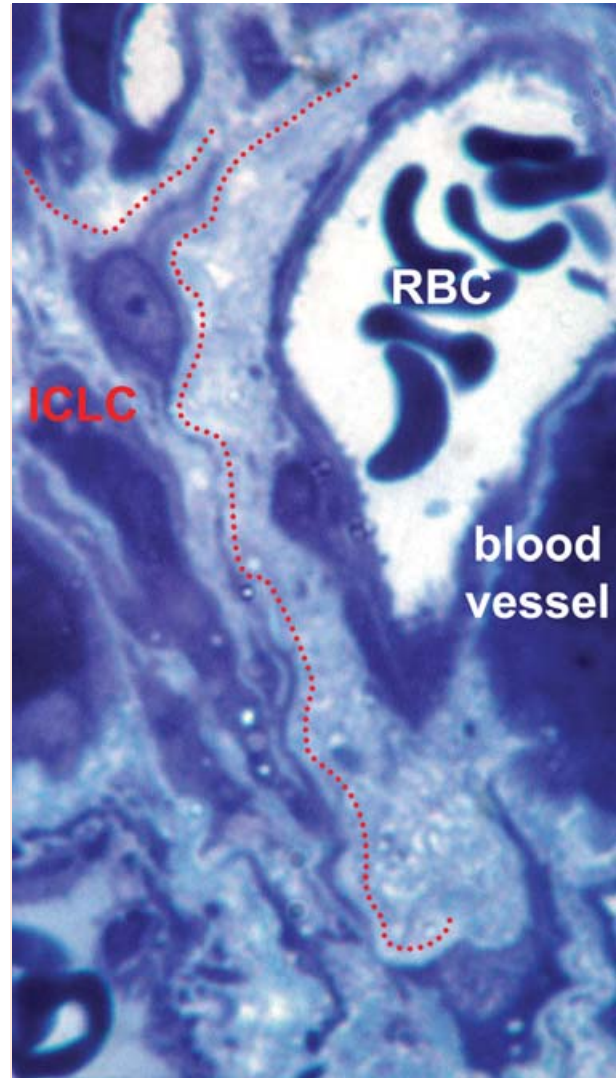

Fig. 4 Light microscopy. Semi-thin section of Epon-embedded chorionic villus stained with toluidine blue. Note the triangular cell body and the very long cell processes. The 'descending' one has more than 50 μm. Ob. 100×.

Donor mothers were in good health and gave their informed consent. The study was conducted in accordance with the moral, ethical, regulatory and scientific principles governing clinical research as set out in the Declaration of Helsinki (1989). This study was approved by Bioethics Committee of 'Carol Davila' University of Medicine and Pharmacy, Bucharest.

As shown in Figure 1, conventional light microscopy (formalin fixation, paraffin embedding, thick sections of several micrometers and H&E staining) is ineffective for revealing ICLC.

Santiago Ramon y Cajal in 1892 used methylene blue staining and silver impregnation when discovered, in association with terminal nerves, or nerve plexuses, the cells known today as ICC.

We used **vital methylene blue** staining on both fresh tissues or cryosections (Fig. 2). Technically, for the vital methylene blue staining, the Shabadash–Niculescu method was applied, as described previously [6]. As expected, ICLC were stained more intensely than the rest of mesenchymal tissue.

In addition, Gomori **silver impregnation** (Fig. 3) showed ICLC in the core of placenta villi. The procedure used for silver impregnation was described elsewhere [6].

Toluidine blue staining of semi-thin sections (0.5–1 µm) is a standardized method for transmission electron microscopy (TEM) in choosing the desired area for ultra-thin sectioning. However, we used this technique to look for ICLC (Fig. 4) since the fixation for electron microscopy is better and the sections are much thinner, allowing the identifications of details not visible in routine light microscopy (*e.g.* because the thickness of ICLC prolongations is at the limit of the light microscope resolving power). Figure 4 illustrates an ICLC with one cell process longer than 50 µm, another one of about 20 µm, but running out of the section plane, and the third being out of the section plane immediately after emerging from the cell body.

Chorionic villi were dissected under the stereomicroscope, mechanically minced into small pieces, digested with 0.05% Trypsin/0.02% EDTA (Biochrom AG, Berlin, Germany) for 1 hr, at 37°C, then collected by sedimentation. The supernatant with any contaminating trophoblast cells was discarded. The remaining mesenchymal core was treated with 1 mg/ml collagenase IV (GIBCO, Invitrogen, NY, USA) for 30 min at 37°C. The resulted single cell suspension and small digested residues were plated in DMEM
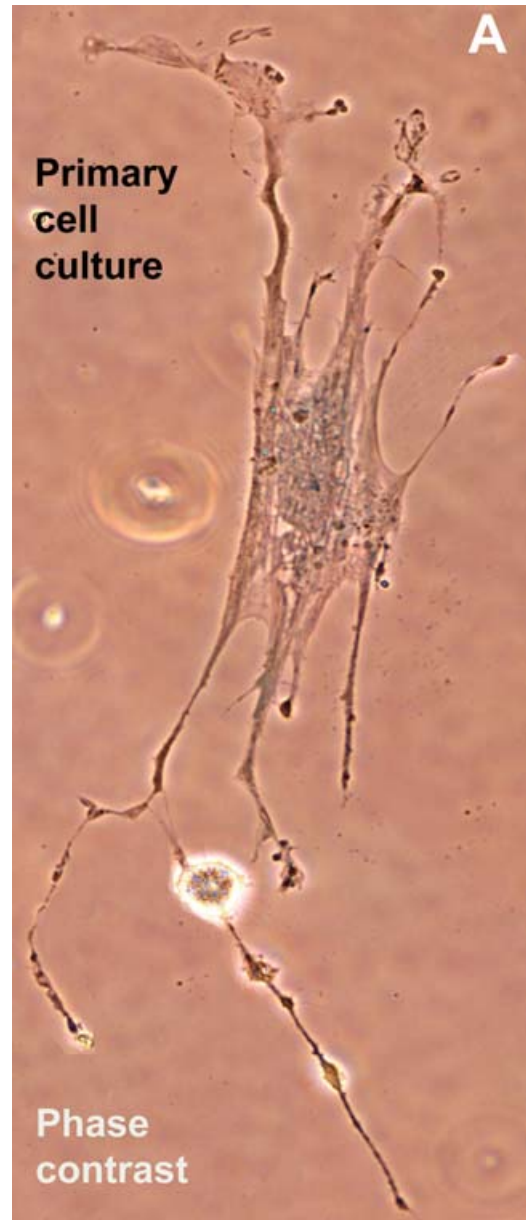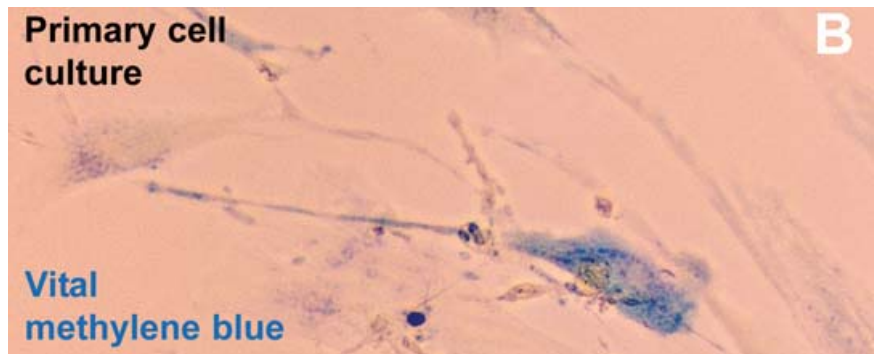
Fig. 5 Human term placenta. Primary culture. **A** *Phase-contrast microscopy* focused on an ICLC with numerous moniliform cell processes. **B** Similar primary culture, light microscopy after *methylene blue vital staining*. Ob. 20 ×, Nikon TE 300.
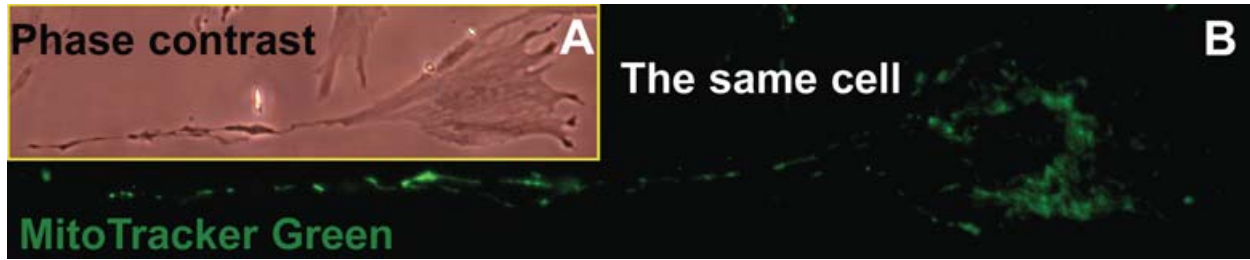
Fig. 6 A and **B.** Human term placenta. **The same cell. A** *Phase contrast* of an ICLC in sub-confluent cell culture (second passage) with a very long, moniliform cell prolongation **B.** fluorescence microscopy after *labelling the mitochondria of living cells with MitoTracker Green FM*. One can observe the 'green mitochondria' concentrated around the cell nucleus and along the cell process, particularly at the dilated portions. Ob. 20 ×.
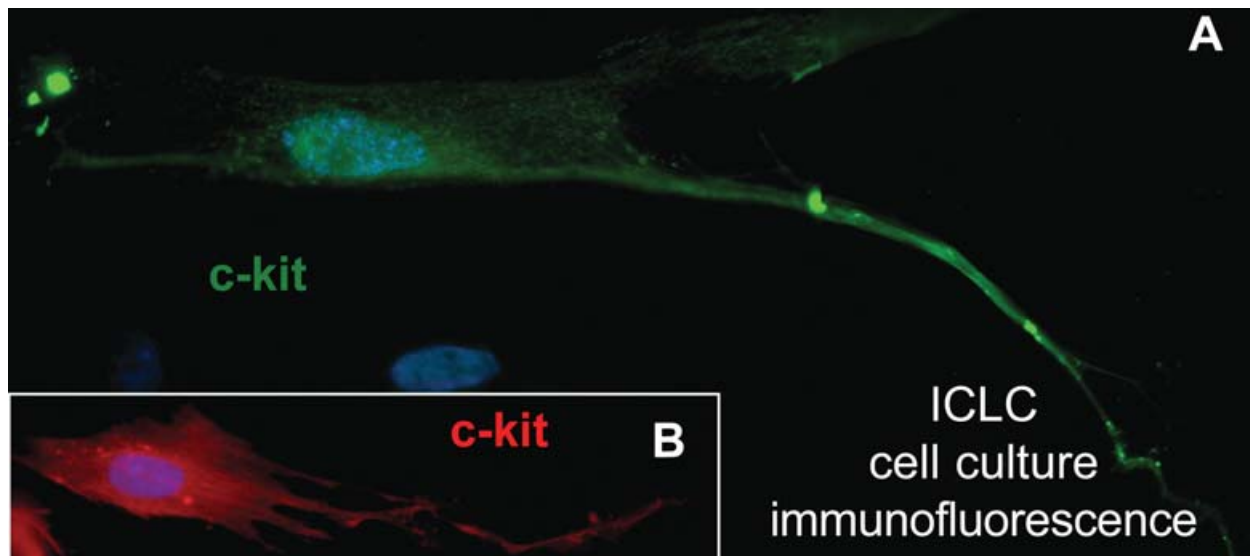

Fig. 7 A and **B** Human term placenta; Cajal-like cells in sub-confluent cell culture (second passage); **Immunofluorescence labelling for CD117/ c-kit. A** *C-kit* localization shown by *green fluorescence*. **B** *C-kit* by *red fluorescence*. Anti-c-kit primary antibodies (monoclonal mouse anti-c-kit, clone Ab81, Santa Cruz Biotechnologies) were detected using secondary biotinylated goat anti-mouse IgG1 (BD Pharmingen) followed by streptavidin AlexaFluor 488 (A) and with AlexaFluor 546 conjugated goat anti-mouse IgG1 antibody (Molecular Probes, Invitrogen) (**B**). Nuclei counterstained with Hoechst 33342 (*blue*). Ob. 100 ×.
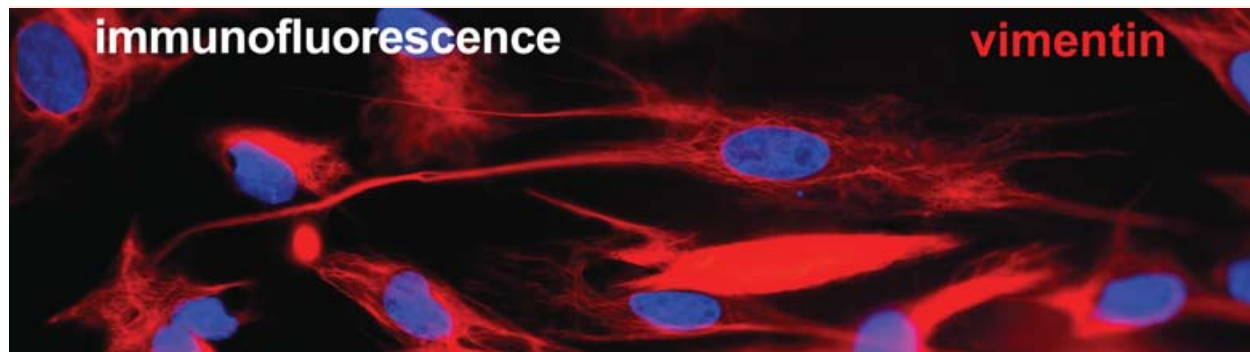

Fig. 8 Human term placenta. Ob. 60 ×. ICLC in sub-confluent cell culture (second passage). **Immunofluorescence microscopy for vimentin**. Cells with morphology of ICLC strongly express vimentin, especially in the cell processes (anti-vimentin Cy3 conjugated primary antibody (monoclonal, mouse, clone V9, Sigma).
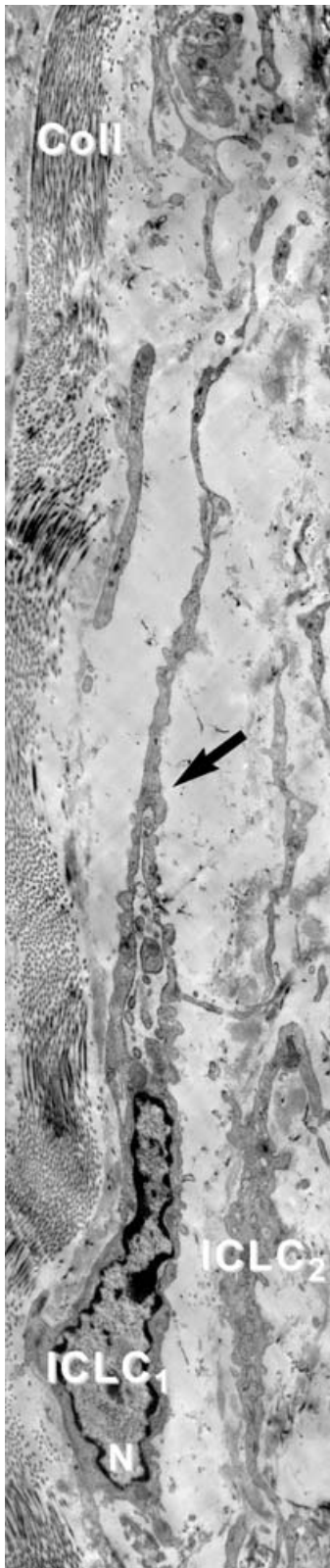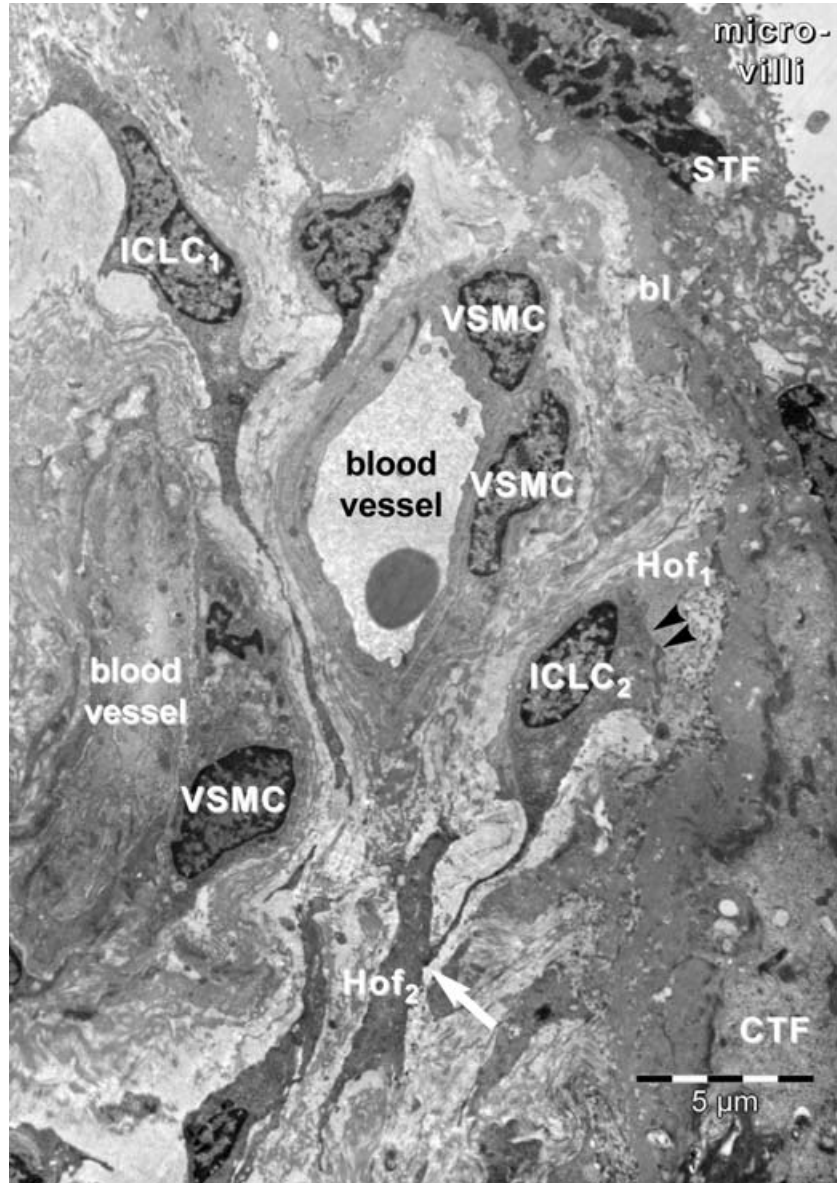
Fig. 11 Human term placenta; TEM of a tertiary villous core in cross-section. An ICLC (ICLC$_1$) with two visible prolongations. The cell is in close contact with two Hofbauer cells (Hof$_1$ and Hof$_2$) at the cell body level (double arrow) and cell process (arrow). Several fragments of ICLC processes can be observed around the foetal blood vessels, one of them also in contact with a Hofbauer cell (Hof$_3$). STF = syncytiotrophoblast, VSMC = vascular smooth muscle cell, RBC = foetal red blood cell.

(Biochrom AG, Berlin, Germany) supplemented with 10% FCS at 37°C, in a humidified atmosphere with 5% CO2. After 5 days in culture, colonies of spindle-shaped cells begin to form around the small explants and in 2 weeks they covered more than 80% of the culture dish, when they were removed and replated.

Figure 5A shows a *typical ICLC* in primary culture, and 5B shows the affinity of ICLC for *methylene blue* (*vital staining*).

Figure 6 demonstrates the presence of mitochondria at the level of ICLC prolongation knobs. The aspect of '*beads on a string*' revealed by MitoTracker Green FM is distinctive for ICLC.

Figure 7 presents (by two different type of experiments) unequivocal immunofluorescence proof, since *c-kit/CD117* expression is commonly accepted as a *specific marker for ICLC*. Mast cells are excluded *ab initio* by microscopical 'design', and fibroblasts or smooth muscle cells are *not* c-kit/CD117 positive. In addition, recent data [11] demonstrated that mesenchymal stem/progenitor cells are c-kit/CD117 *negative*. Figure 8 shows that placental ICLCs express vimentin, which is usually an alternative or supplementary *marker*. Under our experimental conditions (cell cultures) α-SM actin and caveolin 1 appeared positive, as in the case of ICLC from other organs (recent review: ref. [3]). Naturally, caveolin 1 *in situ* appears dominantly in endothelial cells [12].

TEM figures (Figs. 9–11) show the tissular context rather than the ultra-structural details inside ICLC, because the aim of this paper is to demonstrate a distinct population of ICLCs in the multitude of mesenchymal cells of the villous core. Specimens were processed as usually [6–10] and images were taken with CCD camera 2k X 2k Sys attached to a CM12 Philips electron microscope.

Ultra-structural identification criteria for ICLC correspond to the suggested "platinum standard" [6] and we would like to insist on the characteristic prolongations emerging from cell body (see Tables 3, 4 in ref. [3]). Anyway, processes of ICLC from the placenta villi, as well as any other ICLC processes that we have found in various organs [3–10] are the *longest known cellular prolongations in human body,* except some neurons! Even the very recent ultra-structural studies of mesenchymal stromal cells in human term placenta [13] overlook ICLC. In our opinion, ICLCs of human placenta villi are distinct of the so-called 'extravascular contractile system' [14], and are not (directly) involved in human placenta contractions [15].

Obviously, *placental ICLCs* represent a particular case of ICC, since they *do not contact nerve fibres.*

Hypothetically, ICLC of human placenta might perform several roles. Beyond the pacemaker function, which seems to make no sense (at the present time), a role in *intercellular signalling* remains the 'first option'. ICLCs are located in vicinity of small blood vessels or capillaries, in between the 'counter flow' of blood stream and various cells of the mesenchymal axis of villus. Also, their very long cell processes suggest a *juxta- and/or paracrine activity.* As shown in Figures 10 and 11, ICLCs have close ultra-structural contacts (nanocontacts) with immunoreactive cells. Previously, we described such ICLC nanocontacts in various organs [3, 16] and named them *'stromal synapses'* [16]. Stromal synapses represent a new type of synapse, different of the well-known (classical) neuronal or (20-years old) immunological synapse. Moreover, ICLCs appear frequently (Figs. 10, 11) sited as an 'intercellular bridge' between two Hofbauer cells. Thus, a role of ICLCs in *immune surveillance* cannot be ruled out. ICLCs of term placenta villi may function as physical (*mechano-*) *and/or chemical* (*hormonal*) *sensors.* Indeed, we found that ICLCs from human myometrium [17] and fallopian tube [3] express steroid hormone receptors.

Last but not least, why should not placenta ICLCs produce an (un)specific mediator... a *phenomenin* [18]. Anyway, 'placentin' was invoked [19].

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