Numerous papers (including reviews) were recently published about extradigestive localization of interstitial Cajal-like cells (ICLC) [1–8]. In our laboratory we described ICLC in pancreas [9], mammary gland [10, 11], fallopian tube [12] and myocardial interstitium [13, 14]. Moreover, we also found ICLC in human and rat uterus [15, 16]. Hutchings et al. also reported such cells in rabbit uterus [17].

ER and PR are members of the nuclear receptor superfamily together with receptors for thyroid hormone, androgen, glucocorticoid and mineralocorticoid receptors, retinoids, and vitamin D. Progress in cellular and molecular techniques led to the identification of the subtypes and isoforms of estrogen receptors (ER) and progesterone receptors (PR) in female reproductive tract, two for each receptor (ER α and β [18, 19], and PR A and B [20, 21]). Until now, there is no evidence of the expression of steroid receptors on ICLC in situ or in vivo at myometrial level. However, the presence of progesterone receptor A and B isoforms was assessed on human fetal tissues, particularly on interstitial cells of Cajal in the gut, suggesting that progesterone can influence fetal tissue development [22].

Here, we report that human myometrial ICLC in cell culture express estrogen and progesterone receptors: preliminary results were recently presented in another publication [23].
SILVER IMPREGNATION
HUMAN MYOMETRIAL CAJAL-LIKE CELLS IN CULTURE
Material and methods

Human myometrial tissue samples
Fragments of human myometrium were obtained from uteri of non-pregnant (reproductive age) women undergoing hysterectomies for benign gynaecological indications (fragments were excised from areas free of macroscopically visible anomalies). Informed consent was obtained from each woman, in accordance with a protocol approved by the Bioethics Committee of the ‘Carol Davila’ University of Medicine and Pharmacy, Bucharest.

Cell cultures
Cells were cultured using the procedure described in detail elsewhere [15]. The culture medium consisted of Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented by fetal bovine serum (FBS) 10%, HEPES 1.5 mM, 10000 UI/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin (Sigma Chemical, St. Louis, MO, USA).

Staining techniques
Silver impregnation. Cells grown on coverslips, at fourth passage, were fixed with paraformaldehyde 2% for 10 min, at 4°C, washed 3 times in ethanol 80% and incubated for 30 min with silver nitrate 20%. Afterwards, coverslips were washed 3 times in tap water and covered with paraformaldehyde 20% for 3 min and then with ammoniacal silver nitrate solution for 30 sec, washed again in distilled water, fixed in thiosulphate 5% and mounted in Entellan (Merck KGaA, Darmstadt, Germany).

Methylene blue vital staining. Cells on coverslips were washed in prewarmed phenol red-free DMEM and incubated for 20 min, in 0.02% methylene-blue solution, at 37°C.

Slides were examined and photographed under a Nikon 600E microscope equipped with a Zeiss AxioCam MRc5 digital camera.

Immunohistochemistry (IHC)
Cells cultured on coverslips, at fourth passage were processed for IHC. Cells were fixed in 2% paraformaldehyde for 10 min, washed in PBS and underwent the avidin-biotin peroxidase complex method [24]. The primary antibodies used were as follows: CD117/c-kit, polyclonal, 1:400 (DAKO, Glostrup, Denmark), PR, clone 16, working dilution 1:100, and monoclonal mouse anti-human ER, clone 6F11, 1:40 (both from Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). Diaminobenzidine (DAB) was used as chromogenic substrate for PR and ER, and fast red alpha naphtol in Tris buffer for CD117.

Immunofluorescence (IF)
Immunofluorescent double staining was performed on cells cultured on coverslips, at fourth passage. The cells were fixed in 2% paraformaldehyde for 10 min, washed in PBS, then incubated in PBS containing 1% bovine serum albumin (BSA) for another 10 min. Afterwards, the cells were washed again and permeabilized in PBS containing 0.075% saponin for 10 min (all reagents from Sigma Chemical, St. Louis, MO, USA). Incubation with the primary antibodies was performed for 1 h, at room temperature (RT), using monoclonal anti-human PR, clone 16, working dilution 1:100, or monoclonal anti-human ER, clone 6F11, 1:40 (both from Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). The primary antibodies were detected with secondary polyclonal anti-mouse biotinylated antibody (BD Pharmigen, San Jose, CA, USA), working dilution 1:200 and streptavidin-Alexa Fluor 546 (Invitrogen Molecular Probes, Eugene, OR, USA), 1:250. The cells were incubated for 1 h, at RT, with the second primary antibody, polyclonal rabbit anti-human c-Kit/CD117, 1:200 (DAKO Cytomation Glostrup, Denmark), which was detected using a secondary polyclonal anti-rabbit FITC conjugated antibody (BD Pharmigen, San Jose, CA, USA.), 1:150. Nuclei were finally counterstained with 1 μg/ml Hoechst 33342 (Sigma Chemical, St. Louis, MO, USA).

Negative controls were samples without the primary antibodies.

Samples were examined under a Nikon TE300 microscope equipped with a Nikon DX1 camera, Nikon PlanApo 40x and 60x objectives, and the appropriate fluorescence filters.

Fig. 1 Photographic reconstruction of ICLC (**) network, in cell culture, revealed by silver impregnation. Inset displaying the same interfaced distribution of ICLC (**) by methylene blue vital staining. Both methods reveal weaker stained myocytes. Scale bar = 10μm.
Fig. 2 Human myometrial cell culture, fourth passage. Immunocytochemical staining for the estrogen and progesterone receptors. **A.** Immunocytochemical detection of estrogen receptor - dark stained nuclei (*), counterstaining with methyl green for negative nuclei. **B.** ICLC stained positive for progesterone receptor. **C.** Double staining (*) for CD117/c-kit (red) and estrogen receptor (black). **D.** Double staining for CD117/c-kit (red) and progesterone receptor (black). Scale bar = 10 μm.
Fig. 3  Human myometrial cell culture, fourth passage. Immunofluorescent labeling for estrogen (A) and progesterone (E) receptor (red) which appear both inside the nucleus and in cytoplasm. c-kit/CD117 (green) only in the cytoplasm (B, F) and double labeling for both markers (C, G), where co-expression appears as yellow areas. Hoechst 33342 (blue) for nuclear counterstaining. Phase contrast microscopy focused on the same cells, typical ICLCs with long, moniliform prolongations (D, H). Scale bar = 2μm.
Results and discussion

We investigated the myometrial cell culture using silver impregnation and vital methylene blue staining. These methods, applied by Cajal himself on tissue samples, are very convenient for ICLC identification which displayed selective affinity for these dyes. Cell cultures offer the benefit of observing the network distribution of ICLC, myocytes being stained more weakly (Fig. 1). Immunocytochemistry defined, in culture, two cell types in terms of ER and PR immunoreactivity:

a. ICLC showing intense immunostaining, particularly on the nuclei, and weak immunoreaction at cytoplasmic level (Fig. 2 A, B), and
b. myocytes and/or fibroblasts being predominantly negative (Fig. 2 A).

Furthermore, we showed that cells positive for ER and PR were ICLC using double immunostaining for CD117/c-kit, a well known marker for interstitial cells of Cajal (Fig. 2 C, D).

Double immunofluorescence confirmed the same distribution of PR and ER on c-kit positive cells, intense at nuclear level and weak in the cytoplasm (Fig. 3).

The immunohistochemical analysis for ER and PR is frequently used in patients with endometrial or breast tumors in order to establish the appropriate treatment [25–28]. However, little attention was given to normal myometrium, particularly to myocytes, studies being focused especially on endometrium [29] and leiomyomas [30].

A recent study shows that ER and PR expression on human uterine smooth muscle cells in culture significantly decreases with every passage and is much lower compared to cells in situ [31]. This is consistent with our results under in vitro conditions, where uterine myocytes were predominantly negative for ER and PR at the fourth passage.

At this point we can speculate about ICLC involvement in myometrial contractility, during and outside pregnancy, as being steroid hormone ‘sensors’. Exciting new challenges lie ahead involving ICLC as important regulators, which can be eventually modulated with pharmacological agents.

Previous studies state that steroid hormones, especially progesterone, exert an inhibitory effect on gastrointestinal contractile activity [32]. This is particularly true for women during pregnancy [33] and experimental animals under steroid hormone treatment [34]. ICC are responsible for the generation of gastrointestinal contractility, and ICLC in the human uterus also may be involved in a pacemaker role as previously shown [16]. Taking all this into account, the evidence for steroid hormone receptor presence on ICLC might open a path towards the understanding of contractility modulation using steroid hormones in uterus, as well as in the gastrointestinal tract. This effect could be the result of gap junction connections between ICLC and myocytes, and/or the action of paracrine ICLC signaling.

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The female reproductive system as seen by ancient anatomists. Images from Thomas Bartholin, *Verlegung des Menschlichen Leibes* (1677) and *Anatomia* (1673). Courtesy of the Medical Library of the ‘Carol Davila’ University of Medicine and Pharmacy, Bucharest (library catalogue #CS XVII II4 and #CS XVII II3).