Epicardium: interstitial Cajal-like cells (ICLC) highlighted by immunofluorescence

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Abstract

During the last few years, there is an increasing interest in the role of the epicardium in cardiac development, myocardial remodelling or repair and regeneration. Several types of cells were described in the subepicardial loose connective tissue, beneath the epicardial mesothelium. We showed previously (repeatedly) the existence of interstitial Cajal-like cells (ICLCs) in human and mammalian myocardium, either in atria or in ventricles. Here, we describe ICLCs in adult mice epicardium and primary culture as well as *in situ* using frozen sections. The identification of ICLCs was based on phase contrast microscopy and immunophenotyping. We found cells with characteristic morphologic aspects: spindle-shaped, triangular or polygonal cell body and typical very long (tens to hundreds micrometres) and very thin cytoplasmic processes, with a distinctive 'beads-on-a-string' appearance. The dilations contain mitochondria, as demonstrated by MitoTracker Green FM labelling of living cells. Epicardial ICLCs were found positive for c-kit/CD117 and/or CD34. However, we also observed ICLCs positive for c-kit and vimentin. In conclusion, ICLCs represent a distinct cell type in the subendocardium, presumably comprising at least two subpopulations: (*i*) c-kit/CD34-positive and (*ii*) only c-kit-positive. ICLCs might be essential as progenitor (or promoter) cells for developing cardiomyocyte lineages in normal and/or injured heart.

Keywords: interstitial Cajal-like cells • progenitor cells • epicardium • subepicardium • CD117/c-kit • CD34 • vimentin • heart repair–cardiac remodelling

Epicardium is a good example of a 'neglected' component in a vital organ. However, recently, because of the (tremendous) interest in regenerative medicine [1], the epicardium is considered more than a simple 'cover' for heart [2]. Moreover, the epicardium (mesothelium and subepicardium) might be the source of cardiac progenitor (precursor) cells, the key for understanding myocardial repair and/or regeneration [3–19].

In the last decade, numerous publications reported the presence of interstitial cells of Cajal (ICC) outside the musculature of the gastrointestinal tract [20]. We identified interstitial Cajal-like cells (that we named ICLCs) in human and mammalian myocardium [21–26]. Noteworthy, very recently, Faussone-Pellegrini and coworkers [27], based on immune electron microscopy, identified ICLCs in human intestine, beyond the 'classical' ICC.

We present here several images of phase contrast microscopy and immunofluorescence on epicardial primary cell culture, showing canonical ICC (c-kit-positive) and typical ICLCs (c-kit- and CD34-positive cells). In addition, the results were confirmed by immunofluorescence on frozen sections.

Single-cell cultures were obtained from adult C57 black mice treated with 1000 units/kg of heparin. The animals were anaesthesized, and the hearts were quickly removed and transported to the cell culture laboratory in less than 10 min., using ice-cold Hank's buffer Salt Solution (HBSS), pH 7.4, with 10 mM HEPES, penicillin 100 U/ml and streptomycin 100 μg/ml (Sigma-Aldrich, St. Louis, MO, USA). The pericardium was carefully detached from the myocardium, under the steromicroscope Nikon SMZ-2T, and placed in 4 ml of dissociation medium containing 0.125% trypsin (Sigma-Aldrich, St. Louis, MO, USA), 0.025% collagenase II (Biochrom AG, Berlin, Germany) and 0.002% DNase I (Roche Diagnostics, Mannheim, Germany) in HBSS. The sample was incubated at 37°C at 100 rpm in Biosan Environmental Shaker-Incubator ES-20. After 10 min., the sample was removed from the incubator and trypsin was inactivated with an equal volume of foetal calf serum (FCS; Sigma-Aldrich), followed by gentle

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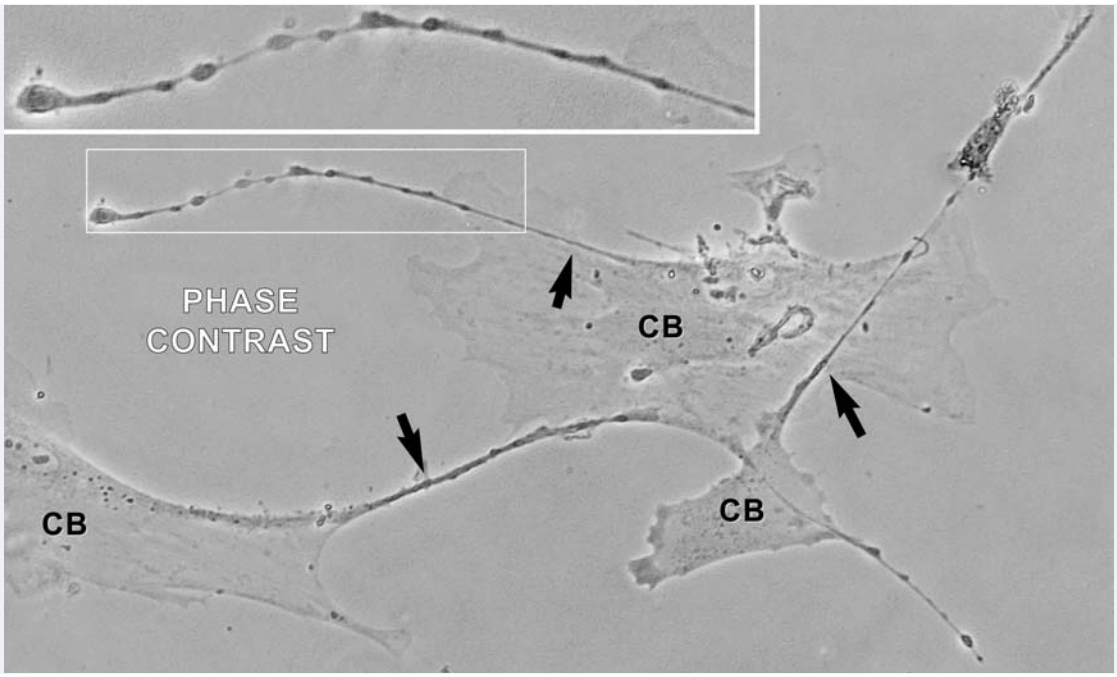

Fig. 1 Mouse epicardium: *phase contrast microscopy*. Interstitial Cajal-like cells (ICLCs) in primary culture (day 5). ICLCs appear interconnected by their processes. The inset shows, at higher magnification, the distal part of the ICLC process with typical 'beads-on-a-string' conformation. Original magnification: 300×. CB, cell body; cell processes, arrows.

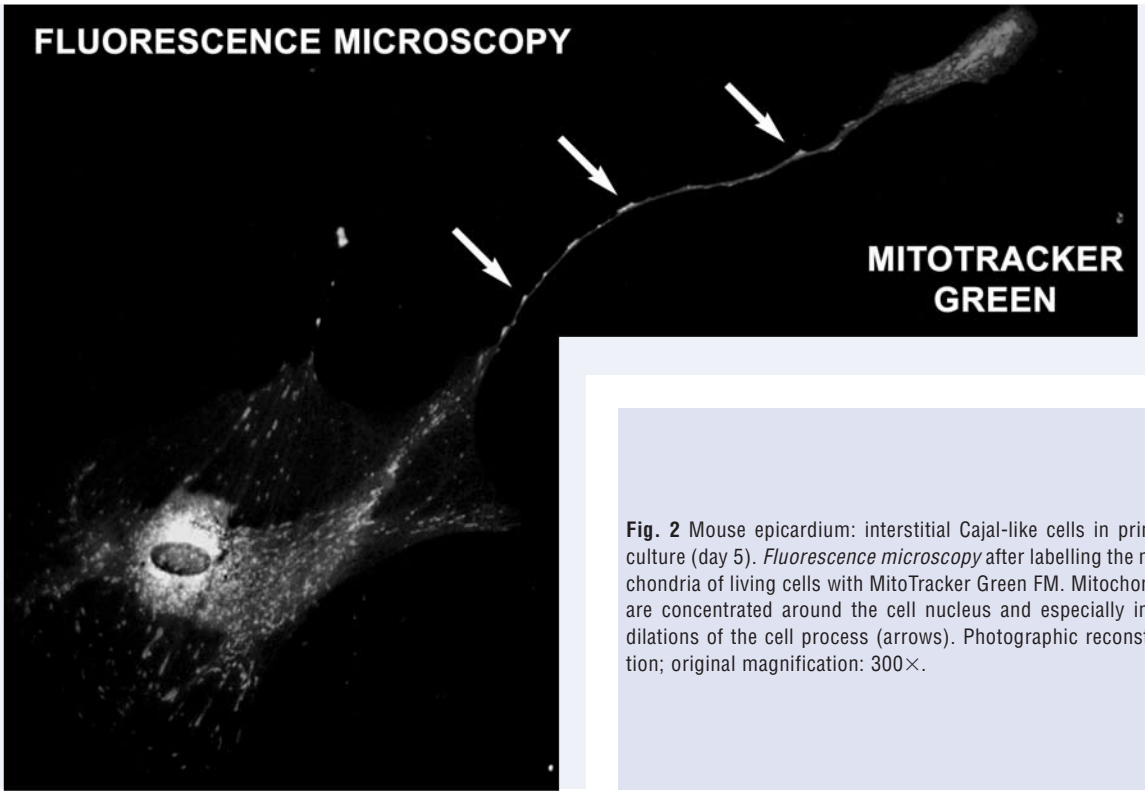

Fig. 2 Mouse epicardium: interstitial Cajal-like cells in primary culture (day 5). *Fluorescence microscopy* after labelling the mitochondria of living cells with MitoTracker Green FM. Mitochondria are concentrated around the cell nucleus and especially in the dilations of the cell process (arrows). Photographic reconstruction; original magnification: 300×.

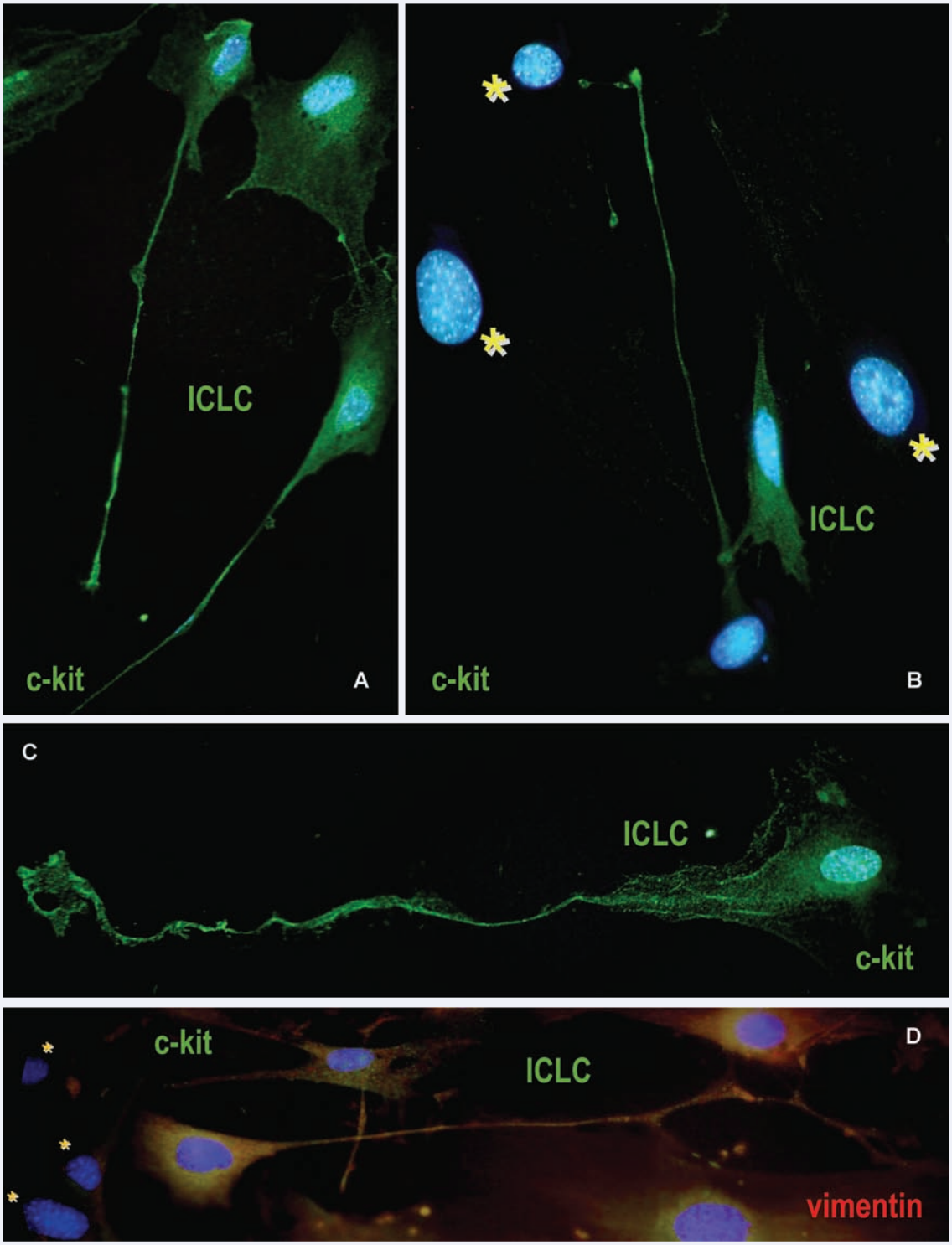

Fig. 3 (**A**–**D**) Mouse pericardium. ICLC *in vitro*; primary cell culture (day 8). (**A**–**C**) *Fluorescence microscopy* clearly shows the c-kit/CD117 positivity of ICLCs (*green*). However, there are also c-kit/CD117-*negative cells*, (*yellow* asterisk). (**D**) Double immunofluorescence staining shows colocalization of c-kit and vimentin (*red*). The nuclei are stained *blue* with Hoechst 33342. Photographic reconstructions; original magnification, 900×.

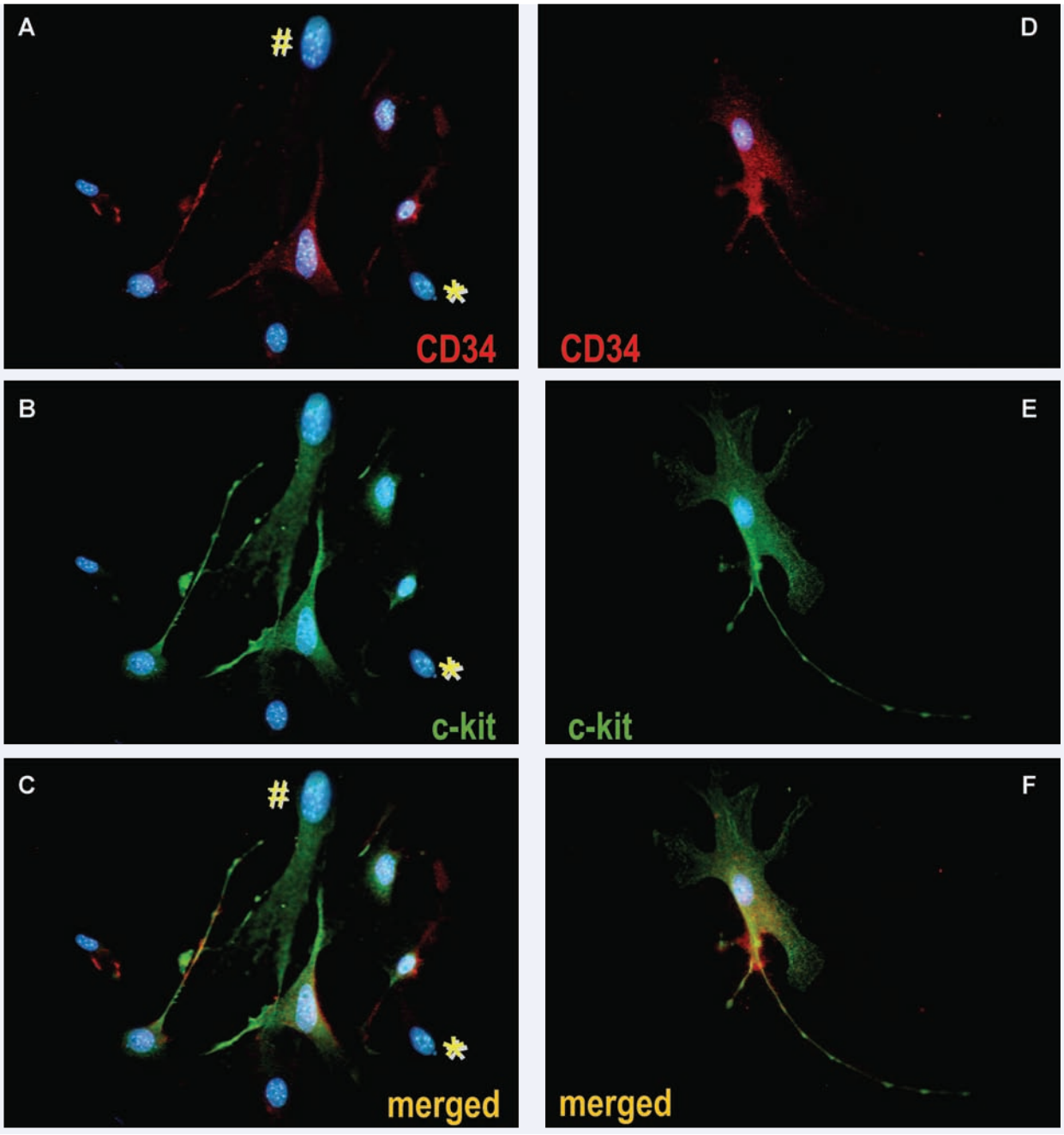

Fig. 4 Mouse epicardium: ICLCs, primary cell culture (day 8). Double immunofluorescence shows that some of c-kit-positive cells (*green*; **B**, **E**) are also positive for CD34 (*red*; **A**, **D**); colocalization appears as *yellow* areas (**C**, **F**). Note c-kit homogenous distribution on the cell surface. CD34 is less expressed at the level of the cell body, but preferentially in the proximal segment of the processes. *Yellow* asterisks designate c-kit- or CD34-negative cells and yellow '#' designates c-kit-positive but CD34-negative cells. The nuclei were counterstained with Hoechst 33342 (*blue*). Original magnification, 900×.

pipetting. The procedure was repeated three times. The supernatant obtained after the first treatment was discarded; afterwards, it was collected, centrifuged and resuspended in ice-cold culture medium with 10% FCS. The remaining suspension with tissue fragments was passed through a 40-mm nylon cell strainer (BD Falcon, San Jose, CA, USA). Each time, the dissociated cells were collected in the same tube and kept on ice. The resulting single-cell suspension was plated on 16-mm glass coverslips

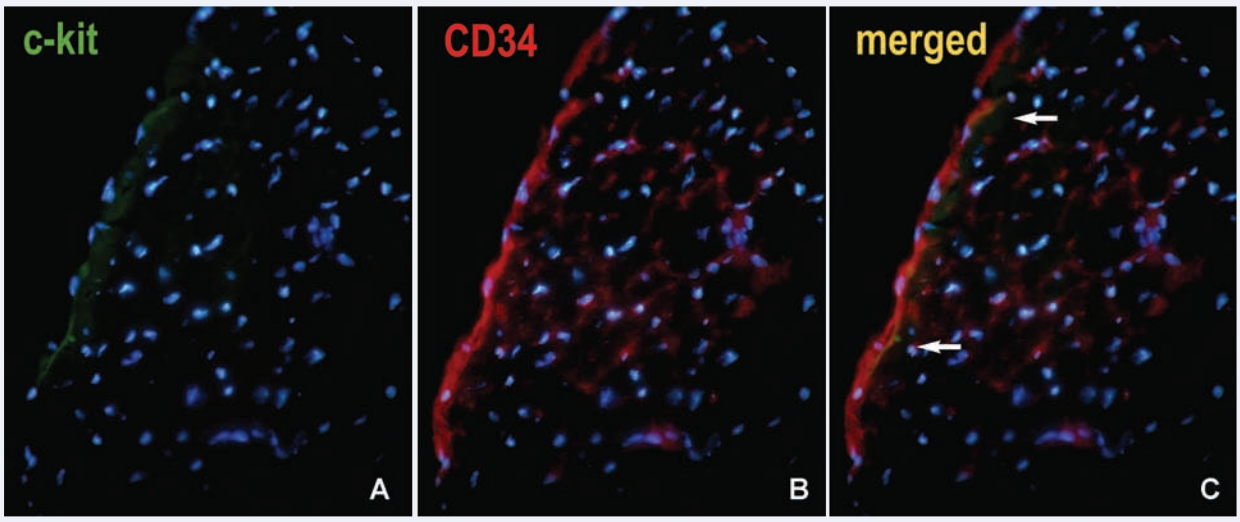

Fig. 5 (**A**–**C**). Mouse heart: frozen sections. *Fluorescence microscopy* shows c-kit- (*green*) positive cells in the subepicardial space (**A**) and a strong reactivity for CD34 (*red*), mainly in the covering mesothelium (**B**). Merged images revealed double-positive cells beneath the mesothelium, as is indicated by the arrows (**C**). Original magnification: 900×.

inserted into 12-well culture plates (BD Falcon) at 37°C in humidified atmosphere with 5% CO_2. The growth medium consisted of DME/F12 (HyClone, Logan, UT, USA), with 2.5 mM L-glutamine, supplemented with 10% FCS, penicillin 100 U/ml and streptomycin 100 μg/ml, and was replaced with fresh media every 2 days. Cells grown on coverslips were incubated with 120 nM MitoTracker Green FM (Molecular Probes, Eugene, OR, USA), a lipophylic, selective dye, which is concentrated by active mitochondria, in phenol red-free DMEM (Sigma-Aldrich) for 30 min., at 37°C, in a humidified atmosphere (5% CO_2 in air). Then, the cells were washed and examined by fluorescence microscopy (Nikon TE200 microscope). Immunofluorescent staining was performed on cells grown on coverslips and on frozen sections. The cultured cells were fixed in 2% paraformaldehyde for 10 min., washed in PBS and then incubated in PBS containing 2% bovine serum albumin (BSA) for another 10 min. In order to label intracellular structures, the cells were permeabilized with 0.075% saponin in PBS for 10 min. (all reagents from Sigma-Aldrich). The frozen sections were fixed with cold acetone for 6 min., allowed to dry, rehydrated with PBS for 15 min. and blocked with 2% BSA for 20 min. Incubation with the primary antibodies (rabbit antimouse CD117/c-kit, C-19, 1:100; rat antimouse CD34, MEC 14.7, 1:50, antimouse vimentin, 2Q1035, 1:50, all from Santa Cruz, CA, USA) was performed at room temperature for 1 hr and they were detected with AlexaFluor conjugated secondary antibodies from Invitrogen Molecular Probes (Eugene, OR, USA). The nuclei were finally counterstained with 1 μg/ml Hoechst 33342 (Sigma-Aldrich). Negative controls were obtained by following the same protocol but by omitting the primary antibodies. The samples were examined under a Nikon TE300 microscope equipped with a Nikon DX1 camera, Nikon PlanApo 60× objectives and the appropriate fluorescence filters. Animal experiments were performed according to the local law for animal protection, with the approval of the ethics committee of 'Victor Babes' Institute of Pathology.

Figure 1 shows typical ICLC morphology, as observed by phase contrast microscopy in cell culture from subepicardial tissue. There is a distinct population of cells with very long (several tens of micrometres), thin (usually less than 0.5 μm), moniliform prolongations emerging from the cell body. As previously described for myocardial ICLCs [22, 23], the dilations contain mitochondria, as indicated by labelling cultured cells with MitoTracker Green FM (Fig. 2).

Figure 3A–C demonstrates CD117/c-kit immunopositive reaction of ICLCs. c-kit positivity is considered as a characteristic marker for the 'true' ICC, described in the gastrointestinal tract [20], as well as an argument for the diagnosis of extra-digestive ICC [26, 28]. A rough estimation of the proportion of positive ICLC-type cells in subepicardial cell cultures indicated ~20%, or even less. Figure 3D reveals the colocalization of c-kit with the mesenchymal marker, vimentin, in cells with characteristic morphology.

Figure 4 provides direct evidence for the existence of two subpopulations of c-kit positive cells: one which is CD34-positive and another which is CD34-negative. About 50% of c-kit-positive cells are CD34-positive too.

Figure 5 presents immunofluorescence results obtained using frozen sections of adult mouse heart. Interestingly, the mesothelium (or under-mesothelium) appears to have a strong reactivity for c-kit and CD34, but CD34-positive cells are

also present in the thin layer of the subepicardial loose connective tissue. Our findings are in agreement with some recent findings focused on the epicardial precursor cells, which suggested that the heart contains a reservoir of cells expressing c-kit/CD117, considered as a stem cell marker [29–31], and also CD34 [10]. However, those studies overlooked the small fraction of c-kit/CD34 double-positive cells. In our study, this population is represented by cells that display ICLC morphology, located just beneath the covering mesothelium. These results are in accordance with current electron microscopy studies in our laboratories [32]. They might represent intermediate stages in the process of epithelial-to-mesenchymal transition, the key element in cardiac repair. Anyway, we previously described by electron microscopy and immunohistochemistry [33] c-kit-positive cells in another serosa: the mesentery.

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