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The face of epicardial and endocardial derived cells in zebrafish

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ABSTRACT

Zebrafish hearts can regenerate through activation of growth factors and trans-differentiation of fibroblasts, epicardial, myocardial and endocardial cells, all positive for GATA4 during the process. A possible model of regeneration of the whole heart and the regenerating cells in ex-vivo culture is presented here by a stimulation of cocktail of growth factors. In *ex-vivo* growth-factors-supplemented culture the heart regeneration was quite complete without signs of fibrosis. Epicardial- and endocardial-derived cells have been analyzed by electron microscopy evidencing two main types: 1) larger/prismatic and 2) small/rounded. Type (1) showed on the surface protein-sculptures, while type(2) was smooth with sparse globular proteins. To confirm their nature we have contemporarily analyzed their proliferative capability and markers-positivity. The cells treated by growth factors have at least two-fold more proliferation with GATA4-positivity. The type (1) cell evidenced WT1 + (marker of embryonic epicardium); the type (2) showed NFTA2 + (marker of embryonic endocardium); whereas cTNT-cardiotroponin was negative. Under growth factors stimulation, GATA4 + /WT1 + and GATA4 + / NFTA2 + could be suitable candidates to be the cells with capability to move in/out of the tissue, probably by using their integrins, and it opens the possibility to have long term selected culture to future characterization.

1. Introduction

In neonatal mice hearts, four cell populations seem to be necessary for regeneration: epicardial-derived (EPD), endocardial-derived (END), perivascular-derived (PD) and cardiomyocytes (CM). Even after the publication of several recent papers that analyze cell populations re-activated after an injury, it is not fully understood whether embryonic-derived epicardial, endocardial and myocytes cells are capable of proliferation throughout life and, assuch, are the sole contributors to the adult epicardium, or if reactivation is restricted to progenitors residing exclusively in the adult heart [rev. [32]]. In the past, the research in mammals has given a great impetus in the “stem cells from cardium” cultivation [10,2,22,23,25,3] with the scope to implement alternative therapies for transplantation to restore the health of patients [30,36]. Although they have found beneficial effects during some experiments with cardiac-derived cultured cells [20], in others there was no evidence of significant improvements in the health of infarcted hearts [20,33]. Recent CRE recombinase-based lineage tracing experiments suggest only a minor contribution to the formation of new cardiomyocytes from such cells in mammals hearts [rev. Chong et al., 2014]. Research of zebrafish have partially explained the failure of the regenerative process by the characterization of these cells as a pool of different ventral mesodermal lineages with specific commitments: epicardium, endocardium, and myocardium ([[17,15,13]). Thus, the restricted capability of regeneration by the cardiac cells seems to be dependent upon the derived lineage and their competence to respond to different morphogenic factors [1,35].

Currently, one of the most interesting approaches is that of translational medicine, which allows the study of mechanisms in organisms with regenerative capabilities greater than those of mammals [1]; rev. [32]. Since zebrafish lineages derived from cardiac cells (CDc) have shown the ability to regenerate the entire tissue, the molecular mechanisms underlying this process lead to cues that point to a translational model, considering the growing data that indicate a strong correspondence between zebrafish and mammals [28]. The discovery that the CDc has shown the ability to turn into an embryonic state of differentiation, in which they express developmental markers [21,27,16,15,13,5] has opened the possibility of studying the differentiation processes. For example, the expression of embryonal markers seems to be dependent on the early down-regulation of key micro RNAs (miR) such as miR1, miR133a and miR133b [28,37,5]. It has also been observed that miRs seem to be responsible in rodents, primate sand fish to establish differentiation and to maintain the differentiation state in the cardiac cell lineages [14,5].

Abbreviations: EPDc, epicardial-derived cells; ENDc, endocardial-derived cells; PD, perivascular-derived cells; CM, cardiomyocytes; CDc, cardial-derived cells; FMs, Fibroblast and Monocytes; FGF, fibroblast growth factor; BME, bovine microvascular endothelial factor; VEGF, vascular endothelial growth factor; PDGF, Platelet-Derived Growth Factor

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After a heart injury in zebrafish, the EPDc seem to have the capability to differentiate fibroblast, perivascular and epicardial cells [35]; rev. [32]. Even with different specialized lineages, during the state of pre-differentiation the EPDc activities expressed the transcriptional Wilm's Tumor factor, WT1 [31,5]. The CM that are close to the damaged area in the heart, re-entered the cell cycle and proliferated from pre-existing differentiated cells and still expressed cardiotroponin [5]. ENDc activation seemed to be restricted to differentiation-potential of the endocardium/endothelial lineage in mammals [40]. Endocardial-derived cells express NFAT2 a transcriptional factor that act by mitogen-activated protein P38 binding and activating the expression of embryonal factors [40]. NFAT2 signaling functions are sequentially activated from myocardium to endocardium. In fact, the early pathway include the blocking of expression of VEGF factor in cardiomyocytes that induce the down regulation of miR133b in both cardiomyocytes and endocardial cells in mammals as well as in zebrafish [40,5,6]. A hypothetical further cell typologies such as fibroblasts and monocytes (FMc) could contribute to the rebuild of cardiac tissue, as has been suggested as result from trans-differentiation of exogenous progenitors that undergo plastic conversion into cells different from the organ of origin [33].

From these multiple mechanisms, the EPDc, ENDc changed receptors and surface molecules and acquired other for moving on tracks of basal lamina components [19,24,38]. All the cardiac activated cells for regeneration express the embryonal marker GATA4 but not c-kit/Sca-1 [17,21,26] very early after the injury [Kikuki et al., 2010]. Some previous studied have pointed the occurrence of a partial activation of epicardium and its regenerative capacity *in ex-vivo* culture of zebrafish heart [39] and of epicardial-derived cells in culture [19]. Although, there are researches that have been used some growth factors as PDGF [39] or thrombin [19], they did not have explored the possibility to use a growth factors cocktail already used in mouse and human cardiomyocytes in *in vitro* cultures by [23]. The aim of this study has been: 1) to produce an *ex-vivo* model of heart regeneration in both whole culture and primary culture of cardiac-derived cells (CDc); 2) to characterize at cytological and immuno-cytochemical levels the cardiac CDc in culture with behaviors of mobility, proliferation, no-adherence. (1) After 1 day (dpa) from the ventricular apex amputation of heart, the zebrafish were sacrificed and the hearts were explanted and cultured for several days in a media supplemented with a GFc and subsequently analyzed at 14 dpa and 30 dpa to assess the eventual actuation of the regenerative program. In parallel experiments, it has been picked the cells that moving out from the hearts already from 12 h and cultured in GFc supplemented media for several days (more that 30 dpa). 2) CDc that have shown the ability to coming-out from heart fragments,remaining no-adherent in the culture plate, were analyzed to assess the morphology by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). At the same time, keeping CDc in culture with with GFc (or without), it has been analyzed the proliferation capability by two different tests (nuclear incorporation of BrdU and cytoplasm permanence of CSFE) in flow cytometry. Finally, the CDc have been analyzed for GATA4,WT1, NFAT2 and cTNT (cardiac troponin-T specific for cardiomyocytes) immuno-cytochemistry. The results have been able to actuate, for the first time, a regeneration model in ex-vivo culture supplemented by GFs, of whole heart and CDc.

2. Methods

2.1. Activation of regenerating program by surgery of heart and ex-vivo culture

The animal experiments were performed according to protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh (US) and Authorization N. 10387/2010 from Italian Ministry of Research at University of Tuscia (Italy). The surgery was performed according to the methodology developed by [27]. Briefly, adult zebrafish (6–18 months old; N = 34) were anesthetized for 3 min in 0.168 mg/l Tricaine (MS222, Sigma) and placed dorsally in a humid sterile sponge, with ventral side up. A small incision (scalpel 0.15 mm, Fine Science Tools Inc. US.) has been made just under the gills, in correspondence to where the heart is located. The pericardial sac has been isolated and punctured to expose the ventricle by gentle abdominal pressure. Approximately 20% of the ventricle apex has been removed by iridectomy scissors (Fine Science Tools Inc. US.). The bleeding has been blocked by gentle pressure with the tweezers for 1 min and the fish has been reanimated by pumped air in water. Approximately the survival of animals with this procedure is 99,7%, and in this research all the zebrafish survived. After 24 h 30 fishes where sacrificed by an overdose of MS222 and the heart has been removed under horizontal sterile flow-hood, washed in Hank's solution added with penicillin/streptomycin 2:100 and used for *ex-vivo* experiments. Four zebrafish has been maintained in tank as a controls animals to evaluate after 14 and 30 days the regeneration grade *in-vivo* and in *ex-vivo*.

2.2. Whole heart in ex-vivo culture for study the capability of the cells to actuate the regeneration by GFs induction-

The hearts extracted has been cultured inwellswith1mleachof cardiac buffer cultures, according tothe following provision: A) L15c buffer (number of animals, N = 15); B) L15c buffer + GFcocktail (GFc, see above; N = 15). The plate was placed in an incubatorFlight ServicePLUS50at a temperature of 28 °C.

The culture buffers has been done by: A) L15 added with 100 × penicillin and streptomycin, 0.5 mg/ml of L-glutamine, 5% Fetal Calf Serum (FCS, GIBCO), 0.8 mM CaCl2 (complete L15 = L15c); B) GFc = L15c spiked with a mixture enriched in GFc constituted by 5 ng/ml of Recombinant Human Cardiothropin-1 (CT, Peprotech Inc.), 2 nM Thrombin (TR, Sigma-Aldrich), 40 ng/ml of Recombinant Human Fibroblast Growth Factor-basic (FGFb, FGF2, Invitrogen, Biosource), 10 ng/ml fibroblast growth factor-4 (FGF-4, Sigma-Aldrich) and 10 ng/ml of Platelet-Derived Growth Factor- BB (PDGF-BB, Sigma-Aldrich) [23].

2.3. Cultured ex-vivo whole hearts of zebrafish

Some of the samples (N = 6 for L15c; N = 6 for GFc) has been maintained in culture for the regeneration studies. Bromodeoxyuridine (BrdU), a chemical that is only incorporated into cells undergoing *de novo* DNA synthesis, it has been added to the fresh media at the concentration of 3.0 mg/ml, during the last 7 days of *ex-vivo* culture of whole hearts (n = 3 for CTRL and N = 3 for GFc). The determination of BrdU has been made with a primary antibody Mouse Anti-BrdU BD Bioscience (Fischer) diluited 1:100 in PBST (PBS, 0,1% Tween) and with a secondary antibody Cy3 diluited 1:500 in PBST, over night at 4 °C. The remaining hearts were kept in culture for 14 and 30 days and then assessed for the degree of regeneration as compare the regenerating *in-vivo* hearts. The Masson's trichrome staining has been done in de-paraffinized sections and treated with Bouin-solution (picric acid/ saturated formalin/acetic acid) pre-warmed to 56 °C for 15 min. The slides have been cooled in running tap water and stained in Weiger ferric hematoxylin (Sigma-Aldrich) for 5 min; subsequently they have been washed in water and treated with a solution Ecarlate Biebrich-Orange G - Acid Fuchsin (Sigma-Aldrich). The slides have been again washed in water and then placed in the solution, F/F (phosphotungstic acid/phosphomolybdic acid) (Sigma-Aldrich) and stained in aniline blue (Sigma-Aldrich). After a rinse in 1% acetic acid have been rinsed in deionized water, dehydrated in alcohol, cleared in toluene and mounted with Entellan. The hearts were observed by fluorescent/light Microscope Axioscope (Zeiss).

2.4. Cell suspension from ex-vivo regenerating hearts

Nine hearts for group (L15c and GFc) has been cultured and maintained for 12 h post explantation (pe). After that period the hearts have been chopped each in four pieces by sterile tweezers to facilitate the exit of the cells and maintained for other 12 h. After that period the non-adherent cells has been collected and used to prepare primary culture. The cells were distributed in triplicate wells of polystyrene plate with 24wells with0,5mleachof cardiac buffers cultures, according tothe following provision: A) L15c buffer; B) L15c buffer supplemented with GFc = GFc. Two wells one for A and another one for B has been added with BrdU. The amount ofBrdU(5-Bromo-2'-deoxyuridine) per well was of 62.5 μl/ml (temperature of28°C and RH85%) for72-hpeand96hpe.

2.5. Flow cytometry with BrdU and CSFE

From the each wells suspension (10^5 per ml) of the BrdU wells were prelevated 200 μl of cells at different time (72 h and 96 h after the beginning of culture) and fixed 10 min on ice with 200 μl of 4% PFA in PBS added with 20% of sucrose. Subsequently they were centrifuged at 1800 rpm for 5 min. The samples were washed with 4 ml of osmolar PBS and again centrifuged. The cells were incubated for 40 min with secondary antibody anti-BrdU, prepared 1:100 in PBS added with 0.5% BSA (Bovine Serum Albumin, Fraction V, Sigma-Aldrich). After a further centrifugation and washing, the cells were incubated for 25 min with fluorescent goat-anti-mouse secondary antibody (FITC-GAM, 1: 200, Cappel). After the routine washing, they were placed in the cuvettes with 250 μl of 1 × PBS for analysis by flow cytometry. The cells placed in the wells without BrdU (A and B groups, at 72 h and 96 h) were directly marked adding 200 μl of CFSE (carbossyfluorescein-Succimidyl- ester) for 15 min. The CFSE is a fluorescentlipophilicmolecule, capable of diffusing freely through the cell membrane. Within cell, the acetate groups are removed by esterase, converting the CFSE in a molecule no longer able to cross the cell membrane. CFSEremainsin the cytoplasm of the cellfor several weeks, and is not detrimental to functionality. The CFSE not can be transferred to adjacent cells. Such characteristics are exploited to study the proliferation by flow cytometry can be followed up to 8–10cell divisions. At the end of the treatment has been collected200μl ofcells, fixed with200μl of4% PFAin PBS20% sucrose for 10 min. Following washes with 4 ml of osmolar PBS, and centrifuged at 1000 rpm, the pellets has been diluted in 250 μl of PBS and analyzed to flow cytometry. Flow cytometry has been used the Epics XL MCL (Coulter) and has being acquired 10^4 events with fluorescence FL1. The analysis has been carried out with the Expo32 software. TheFL1has been set at 512 nm.

2.6. Cytospin with A and B groups of proliferating cells

About 100 μl ofcells were taken from the wells without BrdU or CSFE (72 h and96h), fixed with 4% PFAin PBS + 20% sucrose and centrifuged at 1000 rpm for 10 minutes on slides covered by sterilpoly-L-lisine0.01%(Sigma), driedfor 15 min at room temperature. Once dry, the pellets were washed with 500 μl of PBS for 5 min and mounted with a cover glass by25 μlof PBS-glycerol (9:1). The cytospin suspension were observed with the Axioscope microscope(Zeiss) equipped with image analysis software.

2.7. Immunocitochemistry on cytospin prepared slides

The slides with cells has been dried for 3 h under UV in steril flow in order to diminish the self-fluorence of the cells. The permeability of the cell membrane was has been performed with PBS/Triton 0.25% for 15 min at RT, and then rinsed in PBS 1 × (Na2HPO4 8 mM, KHPO4 1.4 mM, NaCl 140 mM, KCl 1.7 mM). The slides has been treated with ammonium chloride (NH4Cl) 5 nM for 1 h at RT to permanently block cell self-fluorescence. The block of non-specific sites has been performed with PBS/BSA at 2% for 20 min at 37 °C in a humid chamber. Finally, the slides have been incubated with the primary diluted antibody in the 2% PBS / BSA blocking solution: anti-GATA4 1: 100 (Monoclonal rabbit IgG,Abcam); anti-WT1 1:100 (Monoclonal IgG rabbit, Abcam), anti-NFTA2 1:1000 (Polyclonal rabbit IgG, Abcam), anti-cTNT 1: 2000 (Polyclonal mouse IgG,Abcam). The slide have been incubated at 37 °C in a humid chamber for about 1 h. Subsequently, the slides were PBS 1 × washed and then again incubated for 30 min at 37 °C with the GAR 488 (Goat Anti-Rabbit, Abacam 1:500) or GAM (Goat anti mouse, Cappel 1:200) fluoresced antibodies, diluted in DAPI (1 μg/ml). In addition slides have been incubated only with secondary antibody and used as negative controls. Upon completion of the reaction, the slides have been again washed in PB1X and mounted by interposing a drop of glycerol at 80%. Finally, the specimens have been observed at the AxioScope (Zeiss) fluorescence microscope, connected to a computer equipped with a dedicated software for capturing and analyzing images (KS300, Zeiss). Moving was realized by inverted microscope equipped with a JVC camera.

2.8. Electronic scanning microscopy (SEM)

Samples have been prepared at the Interdepartmental Center for Electron Microscopy (CIME)-Center Large Equipment facility of the University of Tuscia. The cells has been collected from the primary culture (100 μl and fixed in 4% PFA for 24 h. They have been subsequently treated with osmium tetroxide 4% in distilled water (1% osmium), 0.1 M sodium cacodylate at pH 7.2, chromium and 1–5% glutaraldehyde for 1 h at 4 °C. Then the samples has been post-fixed in osmium tetroxide 4% in 0.1 M cacodylate buffer at pH 7.2 at 4 °C. After further washing in cacodylate buffer, the samples have been dehydrated using ascendent concentrations of Acetone (from 50% to 100%), then have been cyto-spin on poly-L-Lisine- coverslip and then further dried by the critical point method using liquid CO2 to an apparatus CPD 020 (Balzer). Each dried sample has been adhered to a metal sample holder with conductive double-sided tape and it has been covered with gold using the evaporator Union MED 010 (Balzer). The following observations have been made with a scanning electron microscope JEOL JSM 5200 and photographs have been done through the machine Mamiya applied to the microscope using a film TMAX 100 ASA.

2.9. Transmission electron microscopy (TEM)

The samples have been fixed and dehydrated as mentioned above. The resin infiltration (mixture of Epon) has been performed by using solutions of increasing concentration of acetone and resin in rotor motion(2 rpm/min). Subsequently the samples have been immersed in pure resin to polymerize and putin a stove at 60 °C for 2 days. Included material has been cut with the ultramicrotome Reichert Ultracut with diamond blade. The sections have been collected on copper slides and contrasted with uranyl acetate/lead citrate. Finally, the samples have been observed with the transmission electron microscope JEOL1200EXII. The images have been captured with aCCD cameraSISVELETA(Olympus) through softwarei-TEM.

3. Results

3.1. Whole heart culture and regenerating cells marked by BrdU

Zebrafish hearts cultured with in L15c in *ex-vivo* conditions were able to contract for several months. In culture, fibroblast-like cells has been released from the hearts already from 5 to 6 hpe and were adhered to the culture plate. The hearts after 12 hpe has been anchored to the plate and able to contract for more than 40 days (supplementary data). The regeneration process in *ex-vivo* heart cultures has been shown few capability in L15c cultures and apparently delayed for several days in

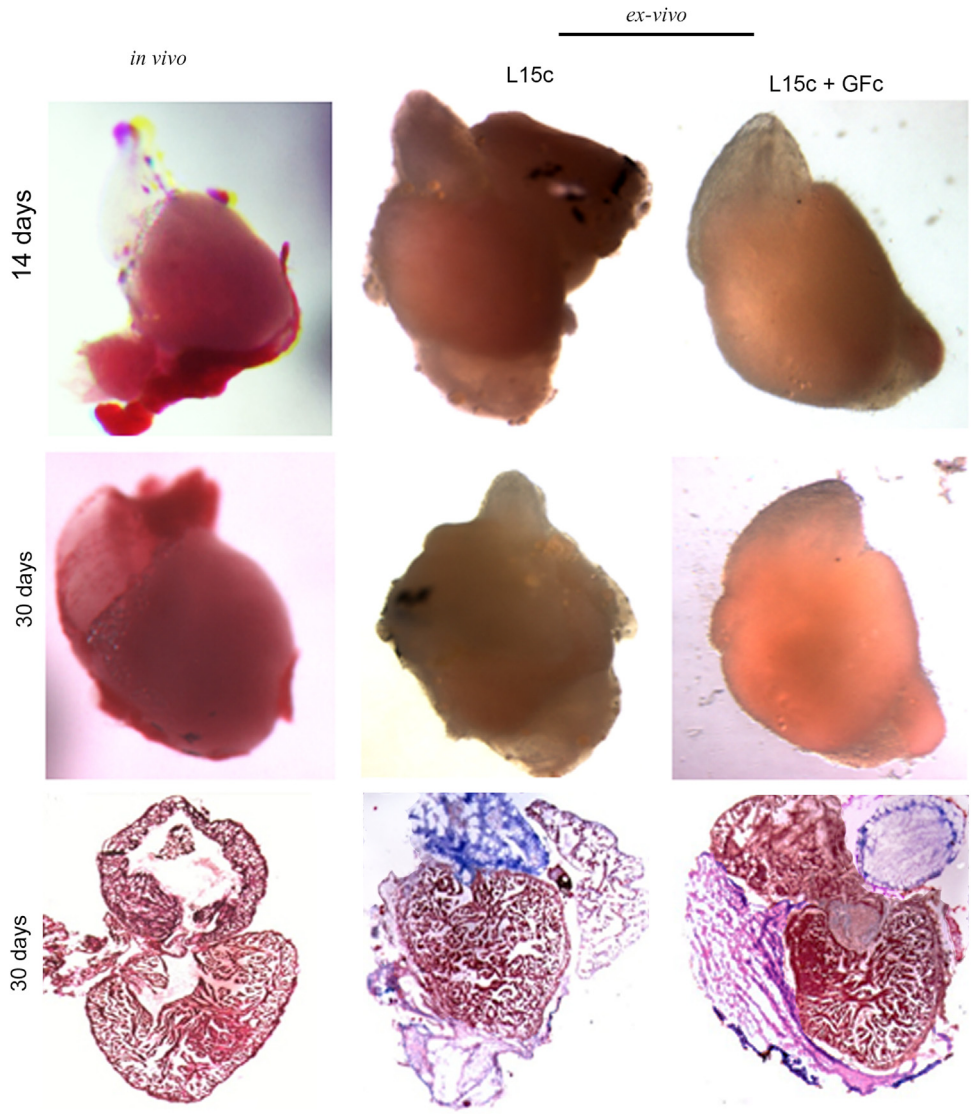

Fig. 1. In-vivo and ex-vivo cultured regenerating hearts. The hearts extracted after 24 hpa has been cultured in ex-vivo with L15 or with L15 added with GFc (right columns) and analyzed after 14 dpa and 30 dpa. The samples has been compared with *in-vivo* regeneration, at the same time. The heart cultured with L15 has not regenerated or has reabsorpted the clot, cleary evidenced in the Mallory's staining in the section (last line). The heart cultured with GFc have shown certain regeneration even if not comparable with the *in-vivo* regeneration. The clot has been almost reabsorbed in GFc like in the control.

GFs supplemented cultures, even not completely comparable with *in-vivo* condition. For example, in L15c a visible diminution of the clot size and the complete regeneration of the ventricle apex has been not observed at 30 days of culture (Fig. 1), nor in 15 days later (not shown). The same culture supplemented with GFc has been shown a great adsorption of the clot area, the absence of fibrosis and a quite comparable regenerated ventricle (Fig. 1, Mallory's staining).

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.yexcr.2018.05.022.

To assess the activation of the regeneration process by *in situ*cell duplication in *ex-vivo* conditions, it has been evaluated the BrdU-cell-labelling in the presence of different media (Fig. 2). The hearts cultured with L15c, evidenced a few activity of cell duplication, especially at 14 days, in all the heart surface (Fig. 2a). An increase of BrdU incorporation from 14 days (Fig. 2b) till 30 days (not shown) were observed adding GFc. According to previous studies in our laboratories, cardiac trans-differentiated moving-cells of zebrafish not show adhesion unless they find a film of fibronectin [Romano N., personal communication] or hyaluronic acid as they move following tracks basal lamina [24]. From the 12 hpe cultured of hearts, the non-adherent cells has been individuated as a elements with light globular (Fig. 2c). By light/fluorescent microscope the organ-culture of the hearts treated with growth factors, have shown an increment in number already after 12hpe (Fig. 2d).

3.2. Primary cell cultures and flow cytometry

Analyzing the forward scatter (FSC) and side scatter (SSC) by flow cytometry (Fig. 3a), the cells that have moved from the portions cardiac showed different sizes and surface: some larger and rough, others smaller and more smoothened of about one-fold less. The culture of CDc with the buffer supplemented of GFc has evidenced an exponential increase number of fluorescent cells (about 2 folds; $n = 200 \pm 50$) by manual counting and after 96hpe (about 6 folds; $n = 200 \pm 20$) than those maintained with the culture-media devoid of factors ($n = 80 \pm 25$; $n = 150 \pm 30$). These data are confirmed by the analysis in fluorescence microscopy of the CSFE cells where it was confirmed the increment of 2–3 folds more in quantity of cells cultured in GFc-cells at T_0as compare with L15c (Fig. 3b, c). Although, both cultures (L15c and GFc) have shown an intense fluorescence at T_0 within

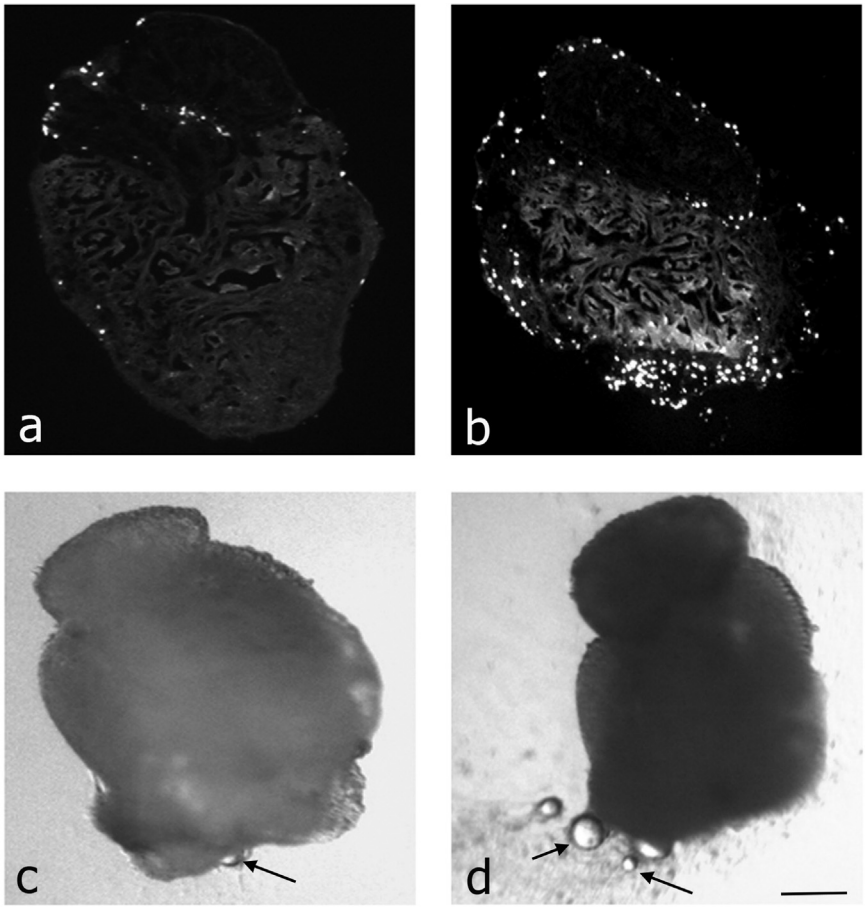

Fig. 2. The regenerating cells has been marked with BrdU and are capable to moving out to the heart. The panel shows the cell duplication occurred in the regenerating hearts 14 dpa-cultured in ex-vivo with L15 (a) or L15 + GFc (b) supplemented during the last 7 days with BrdU. The (a) evidenced a few activity of cell duplication, in all the heart surface; whereas an increase of BrdU in all epicardium portion and close to the clot portion in the heart cultured with L15 + GFc. After already 12 hpa has been observed cells moving from the cultured heart without (c) and with GFc (d) Arrow: smaller and larger cells that moving from the portion of the heart (4×). Bar 500 μm.

R2 (Fig. 3d). After 24 h, the fluorescence of the population was decreased slightly in cultured without growth factors (Fig. 3e), while it decreased significantly in additional crops (Fig. 3f). This result therefore indicates that there has been active cell division that has made more than two folds halve the fluorescence intensity (Fig. 3g). Such an increase in proliferation has been evidenced with similar results also by the other system of BrdU incorporation (Fig. 3h). The CSFE, has been confirmed as a more sensitive proliferation analysis system to use in flow cytometry as compare the BrdU analysis. The cultures incubated at 72hpe with CFSE, and analyzed after 20 min (alias 72 hpe, T_0) and after 24 h (alias 96 hpe, T_{24}) by flow cytometry, have been showed a statistically significant difference between the cells and the T_0 to T_{24} ($P < 0.001$) (Fig. 3g). Furthermore, in T_{24} cells supplemented with growth factors have presented a numerical increase statistically significant ($P < 0.05$) as compared with the L15c-cultured cells.

3.3. SEM analysis

Non-adherent globular cells leak from the portion of the heart and observed in culture has been removed from the plates, fixed and analyzed by SEM after about 96 h (Fig. 4). The cells have confirmed the heterogeneity of size. In fact, we have been observed some larger (type I: 7,5 ± 1,5 μm; N = 25) and other smaller diameter (type II: 4,7 ± 1.5 μm; N = 20). The type I has a cell area equipped with expansions organized in surface-sculptures that may be traced to surface proteins (Fig. 4a, b). It is interesting to note that the type I cells were observed always grouped into elements 2–4 cells. Cell cultures with additional growth factors showed type I cells grouped more compared to those analyzed from cultures without growth factors. The type II cells present, however, a cell surface smoother with sparse surface proteins mainly of rounded structure. These cells are in single or 5–7 grouped elements often showing cell divisions (Fig. 4c, d).

3.4. TEM analysis

The TEM analysis showed at the sub-microscopic nature mobile cell type I and II previously analyzed by SEM (Fig. 5). The type II cells have shown small with high nuclear/cytoplasm ratio and few cytoplasm expansions (Fig. 5a). The nucleus have looked oval with euchromatin in the middle and heterochromatin near the perinuclear cistern. The mitochondria has shown small in size and there were several vesicles with heterogeneous and/or flocculate materials. The cytoplasm also have been presented numerous polyribosomes, RER, different electron-light vesicles, REL and not well organized in an identifiable Golgi apparatus. The type I cells have evidenced larger size and irregular-wavy cell surface (Fig. 5b). The nucleus is regular with chromatin thickened centrally. The cytoplasm revealed an electron-dense nature, filled of organelles: REL, light or dense vesicles, polyribosomes, extensive RER, microfilaments arranged below the plasma membrane. The mitochondria have been of small in size (about 1,3 ± 0,3 μm/ N = 30). Next to these cells, were also analyzed fragments of myocardial tissue placed in culture, to observe the cells that eventually are found in migration. In the samples, they were observed cells which show an epithelial appearance with bundles of intermediate filaments (Fig. 5e). The latter cells had prismatic shaped, with euchromatic nucleus and a nucleolus poorly defined. In the cytoplasm have been evidenced many small vesicles with clear content. Between the cardiomyocytes were observed fragmented portions of pericytes cytoplasmic medium-sized vesicles containing electrons-opaque materials (Fig. 5f). Other large cells (Fig. 5d), have been showed a prismatic morphology with smooth surface. The nucleus has been revealed as oval, large and euchromatic, with the chromatin mostly thickened near the perinuclear membrane.

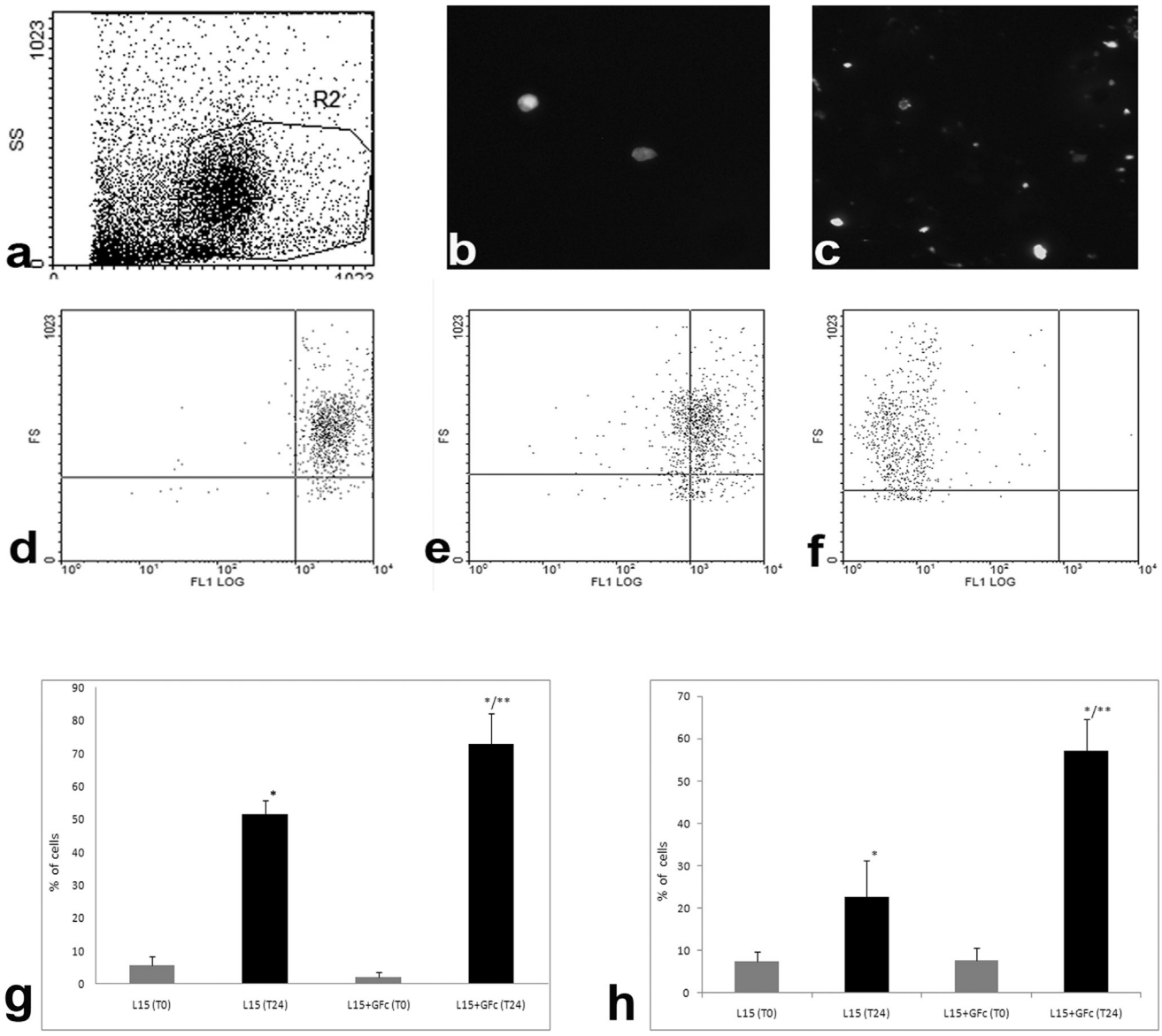

Fig. 3. Flow cytometry analysis and florescence microscopy of regenerating cell in proliferation. a) Dot-plot (forward scatter/side scatter) of all of the cells with the R2 gate enclosing the cells analyzed. b) Analysis by fluorescence microscopy to T24 of cultured cells without GFc. c) Analysis by fluorescence microscopy to T24 of cultured cells with growth factors. Flow cytometry analysis of the proliferating cells at T0 (d) and at T24 with non-supplemented (e) or supplemented with GFc (f). Note the plot in (f) where the proliferating cells are at least two folds and of larger dimensions. FS = forward scatter; FL1 (fluorescence of CSFE); SS = side scatter. Cultured cells incubated 72 hpe (T0) e 96 hpe (T24) with CFSE (g) or BrdU (h) to evaluate the proliferative index of non-adherent cells. (*) T0 vs T24 ($P < 0001$); (**) L15c (T24) vs L15c + GFc (T24) ($P < 0,05$).

The nucleolus has been also evidenced. The cytoplasm has been shown as enriched in vesicles and REL and not obvious bundles of intermediate filaments or microfilaments has been evidenced.

3.5. Immunocytochemistry of CDc in culture

Immuno-cytochemistry of the cells cultured with GFc revealed a large amount of $GATA4^+$ cells of both typology small and large (Fig. 6). $WT1^+$ cells have been only of large prismatic type, whereas NFAT2+ cells have been only the small round type. Cardiac Troponic fraction T (cTNT) antibody not reacting with this type of cells.

4. Discussion

In recent years, zebrafish have become an important model system for science [20]. New mutant and transgenic lines are constantly emerging and publications using this model are biological increasing exponentially [4]. The peculiar feature of the zebrafish to regenerate different tissues, such as heart, allowed to promote it as a model system to understand the main molecular and cellular mechanisms that underlie the block in the regeneration of the mammalian heart. In this scenario, zebrafish might possibly be on the same level as mice in the near future. However, there are still some deficiencies that need to be overcome, particularly the establishment of turn of embryonic state of heart cultures. During zebrafish heart regeneration, cardiomyocytes in the cortical layer of the ventricle induce the transcription factor gene *gata4* and proliferate to restore lost muscle [13,16]. A dynamic cellular mechanism initially creates this cortical muscle in juvenile zebrafish, where a small number of internal cardiomyocytes breach the ventricular wall and expand upon its surface [21]. Currently cardiac stem cells in mammals have been poorly characterized morphologically except through markers c-kit / Sca-1 [12,23,3] and in fish only indirectly

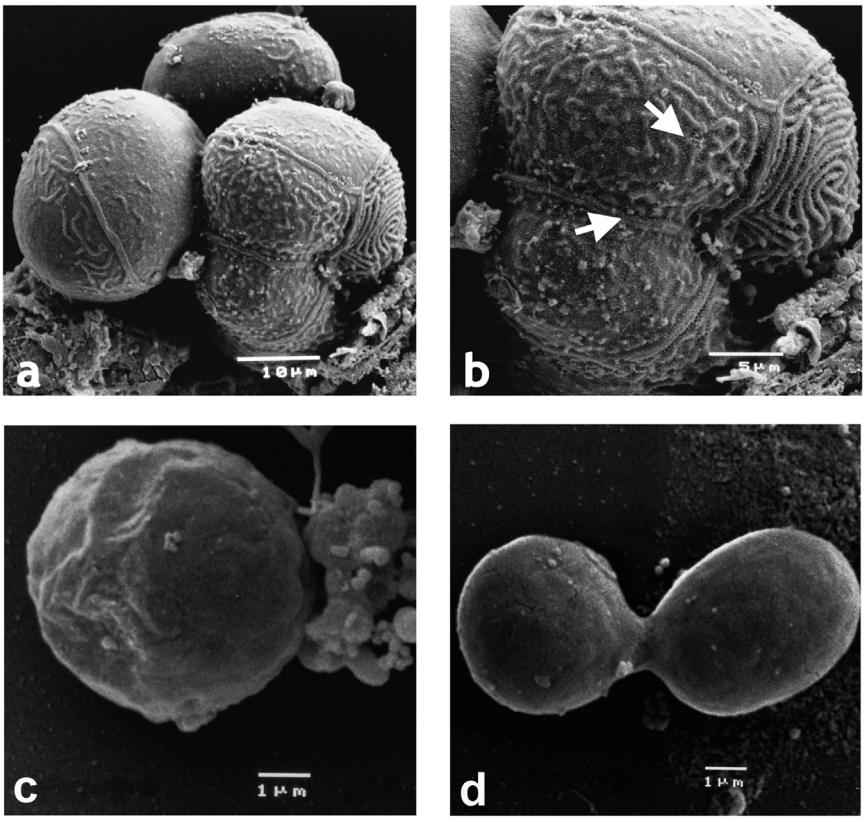

Fig. 4. SEM analyses of regenerating cells. a) by SEM analysis of cells spills from the heart and cultured for 96 h Showed variously rippled surface (magnification 2000×). b) Detail of photo (a) where there are clear proteic-sculptures on the plasma membrane of these cells (arrows; 3500× magnification). c) Some cells show small rounded and smooth surface with sparse rounded proteins (magnification 10,000×). d) Cells similar to those in c) that highlight cell divisions if maintained in buffer supplemented with FGF-cocktails (magnification 10,000×).

by Lepilina [21] that assessed expression of islet1, islet2, and islet3, as well as a-kit and b-kit, during heart regeneration but no c-kit. It is common practice to assume that markers associated with embryonal/pluripotency in other species can be directly extrapolated to zebrafish [1], even if some differences can be reported. For example, in zebrafish the homologue of c-kit is a marker of melanocytes but not of cardiac stem cells [26]. Aiming to increase knowledge on the morphology of cardiac regenerating cells, it has been actuate a primary culture of regenerating-hearts and CDc in *ex-vivo* cultures. The aim has been actuate a protocol of long term expansion of CDc and/or a organ-model of regeneration in *ex-vivo* of heart. Moreover it has been tried to do a cyto-morphological characterization of CDc, in order to complete the knowing about these cells. The protocols in this research have been developed independently from what described by [4]. These authors, similarily to our experiments, have actuated a tentative to culture in *ex vivo* the hearts and cells in a L15 medium. Although they have some success in studying the epicardial cells, they, as well as we have here reported, they not obtained regeneration. Thus, they have hypothesized that the absence of GFs has been the cause of the in success [4]. In our research, we have worked for months to calibrate the correct cocktails of GFs to obtain the best expansions of cells or heart regenerations (data not shown). The cocktail described in the present manuscript is, at moment, the best mixture of GFs to regenerate the hearts in *ex-vivo* culture and to expand the CDs for months. Thus, we decide to characterize the ex-vivo culture cells. A first analysis to the light microscope showed, already after 12–24 hpe, the presence in organ-cultured of two types of non-adherent cells of different sizes: the type I has major dimension as compare the type II. Similarly, other authors have described the capacity to migrate out of the heart in zebrafish [19] and seems to be a common process also in explants of mouse and human [23,8]. These cells showed an increase in size going from a few microns to a 20–100 µm; they have also shown the ability to aggregate in cardiospheres after 10 days of cell culture by exposing cadherins [23,7,8]. Among the moving out cells, fibroblast have been responsible to the explants adhesion and, later on of the cardiosphere formation [8]. Thus, the ability to left the explants/damage area seemed to be common in both zebrafish and mice. The CDc has been coming from different heart portions:ventricular cardiomyocytes [15,21], endocardial or vascular [20,4] epicardial regions [21,24,5] and connective fibroblasts [1,19]. By using GATA4 antibody we have evidenced the positivity in all typologies. Moreover, the antibody against the Willem Tumor 1 (WT1), specific for epicardial cells in pre-differentiation [31], has permitted the characterization of the type 1, as a epicardial element. In meantime, the antibody against NFAT2, specific for endocardial-derived cells [40] has been defined as the type II. At the moment, due to the culture system, that excludes fibroblast and trans-differenziate myocardiocytes (cTNT-positive), we can hypothesize that the origin could be from epicardium (type I) and also by endocardium (type II).

Another question mark could be why we are sure that the proliferating cells are the reparative stem cells of the zebrafish heart. [21] have shown that proliferation of cardiac stem cells of zebrafish has been mainly due to growth factors: in the ventricle injured area, the adult cardiomyocytes/fibroblasts have released growth factors (like FGFs) which, captured by FGF-receptor on epicardium cell, are able to activated the motility by expression of hyalauronan-receptor [24] and proliferation into the amputated area [15,34]. It is recently know that overall FGFs, several factors are involved in development/regeneration of zebrafish heart, such as hedgehog [11], insulin growth factor and TGFβ [9]. However, particularly PDFG and retinoic acid seems to be specific for epicardial activation [17,18]. For this reason, in this research in the GF-cocktail was added, a part different FGFs, also the PDFG and BME factors. To compare the degree of proliferation between cells cultured with or without growth factors, flow cytometry analysis has been performed after 72 and 96 hpe. This experiment was carried out in parallel by incubating cells with either the intercalating-BrdU of DNA, both with the lipofilling-fluorescent molecule, CFSE. It observed a clear cell proliferation, with a great enhanced in cultures supplemented with GFs; This suggests an effect of stimulating proliferation by these

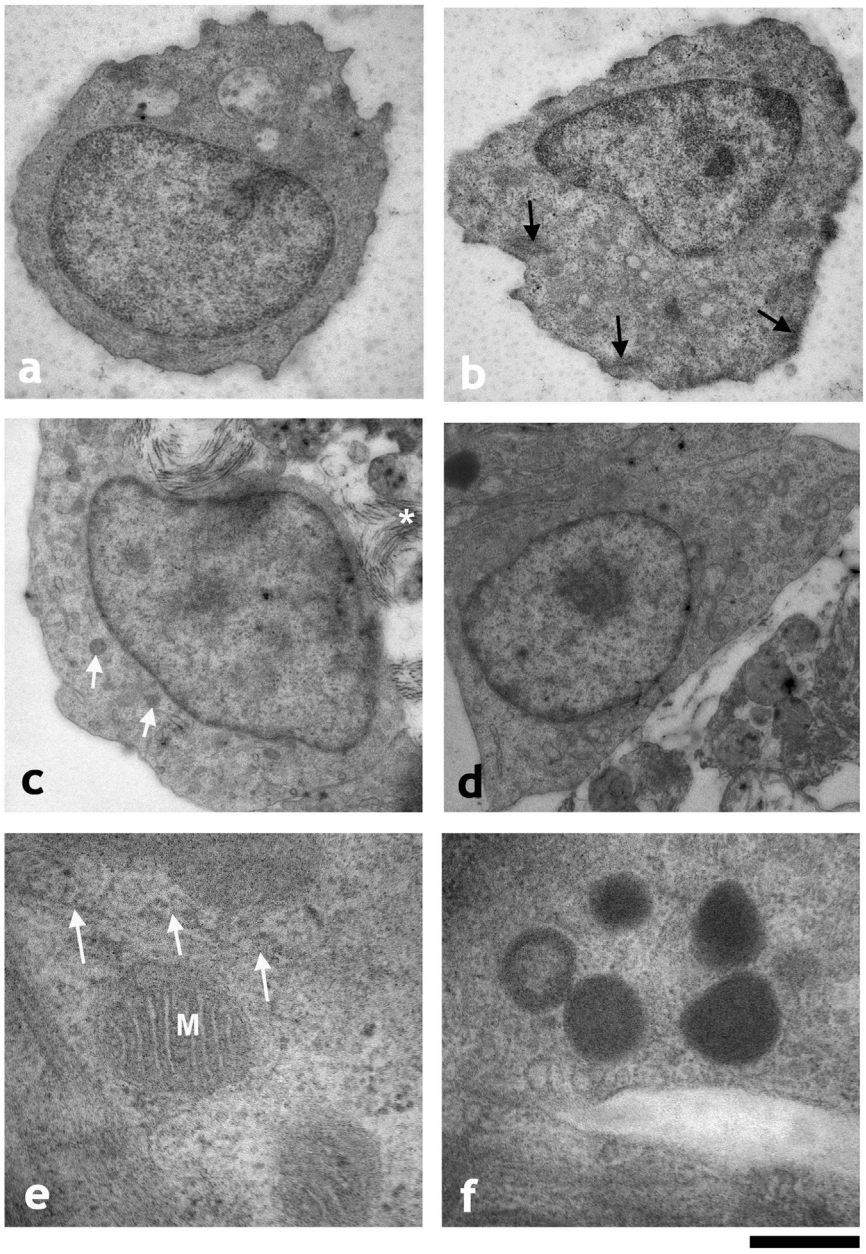

Fig. 5. TEM analysis of cardiac regenerating cells. a) Cell from the cultures of type II, that show oval nucleus, electron-dense cytoplasm with numerous organelles. (Bar. 0,8 μm). b) Cell of type I show a prismatic nucleus and a cytoplasm enriched of vesicles. Arrows: actin microfilaments (Bar: 2 μm). c) endocardial cell from the heart fragment evidences a prismatic nucleus. (Arrows: vesicles with content is clear that dark; *, Collagen fibers; Bar: 1 μm). d) Cell flaking from a fragment of cardiac tissue. It show a prismatic morphology and a large oval nucleus. (Bar: 0,8 μm). e) M: mitochondria with mitochondrial membranes and crests belonging to pericytes cells observed in fragments of the myocardium (arrows: bundles of intermediate filaments; Bar: 1 μm). f) vesicles medium sized opaque to electrons, observed in pericytes located between muscle fibers fragmented (Bar: 200 nm).

factors on CDc and that our cultured cells are sensible/competent to that GFs as described above in zebrafish by our group [24]. The data, inedited in the references, about the cellular heterogeneity of the typesI and II, was revealed by light and electron microscopy analyses. The SEM analysis has been confirmed the morphological information obtained by the flow cytometry and TEM analyses: the typeI has a greater diameter, a surface provided with protein-like protrusions arranged in sculptures; the typeII has a smaller diameter and a cell surface smooth or slightly wrinkled with sparse rouded-protein-like protrusions. [23] have shown that murine cardiac stem cells, organized in cardiospheres possessed surface sculptures similar to those that we have described as type I. At the same time the work in mouse of [3] have shown the presence of N-cadherin, fundamental for the formation of desmosomes between cardiomyocytes, and cytoplasmic proteins such as connexin43 [29]. These data from other species, have suggested that probably the sculptures cellular highlighted in type I cells might be surface cadherins. However, further biochemical studies are needed to confirm this hypothesis. The TEM analysis have evidenced the cytology of the cells collected from culture medium supplemented with FGFs cocktail. Both typologies have showed a more dense cytoplasm with numerous mitochondria organelles of large, suggesting an intense cellular metabolic activity. Moreover, high number of mitochondria observed in cardiac stem cells of type I than type II. Even if no data are available in zebrafish, an indication can be exerted by [12] in humans. They have highlighted how there can be a close match between the advanced stage of cellular differentiation and the presence of a quantity of organelles and above all a large number of mitochondria in the cytoplasm of cardiac cells. Further immunocytochemical analysis has pointed that the type I cell has been shown also a positivity to WT1 and GATA4 markers. The WT1 is a marker of epicardial-derived cells in zebrafish as well as in mice and humans [32], thus we can ascribed the type I as EPDc. The type II, has shown positivity to NFAT2/ GATA4 (and negativity to WT1 and Troponic C). Since NFAT2 is a marker of endocardial-derived cells, and possess characteristics described in human endocardiocytes [41], we can hypothesize that the smooth and small type II is a ENDc.

In conclusion, for the first time there has been demonstrated the capability of GFc to lead a heart regeneration in *ex-vivo* by activating

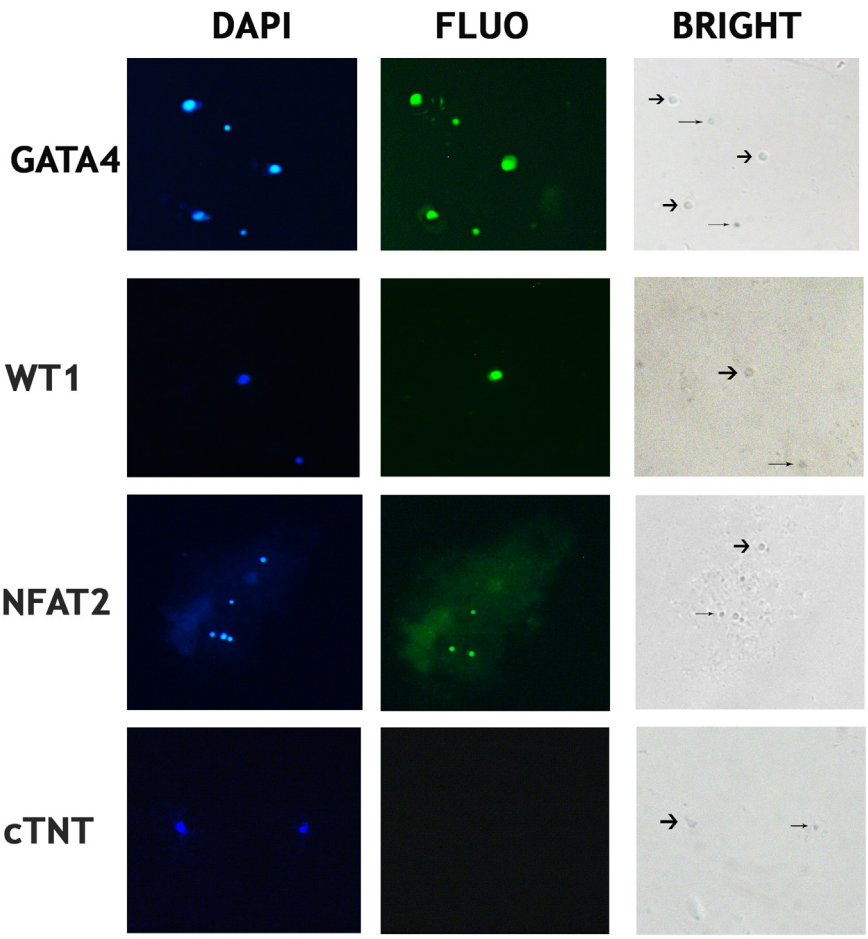

Fig. 6. Immunocytochemisrty with GATA4, WT1, NFTA2 and cTNT antibodies of 72 h-proliferating cells. GATA 4-green/DAPI-blue + cells are of both larger (type I) and small (type II) dimensions, also detectable by bright light picture. WT1/DAPI + epicardiocytes are only of larger type. NFAT2/DAPI + endocardicytes are only of smaller type. cTNT/DAPI evidence a negative stained, demonstrated that the proliferating cells are not cardiomyocytes. Big arrow: type I cells; small arros: type II cells. (magnification 40 ×).

the regenerative cells. Moreover, the ex-vivo cultures of no-adherent cells have been permitted a morphological. immunocytochemical characterizations of CDc, that can be ascribed to epicardial (larger type II) and endocardial (the smaller type II).

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