METHOD FOR THE ISOLATION OF SUBPOPULATIONS OF CARDIAC PROGENITOR CELLS AND RELATED USES IN THE MEDICAL FIELD

The present invention relates to a method for the isolation of subpopulations of cardiac progenitor cells from a heart tissue sample, the population thus obtained and the related uses in the medical field for the cell therapy or cardiac cell and/or tissue transplantation field.

Progenitors are all cell populations that have the ability to proliferate and differentiate, also performing a support function for surrounding cells and tissues.

Progenitors are rare cells located in the tissues of living beings in order to perpetuate their functionality through the exchange of damaged cells. They were initially discovered in tissues with the highest cell exchange such as bone marrow and epithelial tissue, but it is now well known that there is a population of progenitor cells living in different tissues, also with reduced regenerative ability.

The discovery of the progenitors resident in the adult heart has revolutionized the dogma of the heart as an organ incapable of self-renewal.

This discovery has led to studies on resident cardiac progenitors with the aim of developing therapy able to regenerate the myocardium after ischemic or other damage. Different types of cells, characterized by different surface markers have been proposed and studied and some have been tested in phase I, and some phase II, clinical trials initially approved by the FDA in the USA and more recently also in the rest of the world, but not in Italy as yet.

Such knowledge of cardiac progenitors has widened research horizons in order to introduce innovative therapies within what is defined as regenerative medicine.

The use of stem cells (including resident cardiac progenitors) for clinical trials must follow rigorous criteria. They ensure that the translation of stem cells is performed according to the same regulatory standards used for the production of medicines.

Good Manufacturing Practices (GMPs) are guidelines for the production of medicines according to the current reference pharmacopoeia (American, European, etc.). In Italy, the phase I clinical trial for cell therapy products (CTPs)

is subject to the prior opinion of the Italian Institute of Health (ISS). From the perspective of possible clinical use of cardiac progenitors it is therefore fundamental to develop and validate the procedures for preparing them with reference to the GMPs in accordance with the 2004 guidelines of the ISS on CTPs.

The isolating protocols available in the art (see ref. [4], [5], [6]) allow isolating cardiac progenitors populations ($CD117^+$) using a magnetic selection system to research scope. In fact, in order to select the population of cardiac progenitors using the magnetic selection system for GMP productions and clinical trials it is necessary first of all to extensively expand the unselected starting population whose selection is desired. The selection system having research scope allows the selection starting from a number of cells of about $1x10^6$, $1x10^7$, while the GMP-grade selection system which is nowadays available is not working starting from such a small number of starting cells. In order to make the immunomagnetic selection system working it is necessary to select a starting cell number of about $1x10^9$ (peripheral or marrow blood cells), that is the first mandatory condition to select a progenitor population under GMP-grade conditions is adopting a strategy to maximize the selectable starting population approaching the amount of $1x10^9$ cells.

Therefore, to date, there is no method on the market for the production of cardiac progenitors that can be used in clinical trials in Italy where the ISS already requires GMP production levels for phase I studies, like the legislation for the production of medicines.

From the perspective of the cell therapy in the cardiovascular field, the cell type covers a fundamental role for therapeutic success. By analysing the cardiac tissue as a source three different types of cells have currently been proposed, already tested in phase I clinical trials and some in phase II.

In particular:

1) the population of cardiac stem cells (CSCs), selected for the surface expression of CD117 antigen, c-kit [1];
2) cardiosphere-derived cells (CDCs), not isolated by a marker, but for their

ability to form spherical aggregates [2];

3) phase I studies and trials that use a combined therapy approach through the use of a combination of CSC-ckit^{+} cells and bone marrow mesenchymal stem cells (MSCs) [3].

All three types of cells listed above have advantages and disadvantages according to the type of cell and the manufacturing technique that allows them to be obtained.

For example, the cardiosphere-derived cell population not selected by any marker (CDCs) has the advantage of being easy to obtain in large quantities, but the disadvantage of being a heterogeneous population that is not clearly characterized.

On the other hand, the CSC population has the advantage of being enriched with very well characterized stem cells/progenitors and the proven therapeutic function in terms of functional increase of the myocardium and the quality of life of the patients treated; however, the aforementioned population displays the disadvantage of being an extremely rare endogenous population, with reduced proliferation capacities therefore difficult to obtain in a clinically significant number.

The latest approach currently available (combination of CSCs and MSCs) could potentially have a better therapeutic advantage as the combinations of cells could produce better results, but at the same time the mechanism underlying the possible improvement remains unclear; furthermore, the cell combination introduces variability in the determination of the efficacy ratios and the most active population in the damaged myocardium.

The authors of the present invention have now developed a method for the isolation of human cardiac progenitor cell subpopulations (hCPCs) that allows the different technical problems affecting the isolation processes of the background to be solved, particularly in terms of efficiency in obtaining a clinically significant number of hCPCs and high purity standards.

In fact, the method for the isolation of cardiac progenitors according to the invention allows different subpopulations of cardiac cells to be expanded in a controlled way from the primary culture, maintaining their phenotypic

characteristics and obtaining a clinically significant number of cells in rather reduced time frames.

Another objective of the method according to the invention is that of isolating cells from any biopsy coming from any heart source (solely by way of example, the left and right auricle, septum, apex, ventricular biopsy) even starting from a tiny amount of starting material.

The isolation method according to the invention is also advantageous as it is extremely versatile, i.e. adapted for the selection of any subpopulation present in the original population that positively and/or negatively expresses a surface antigen, also in GMP conditions. GMP (Good Manufacturing Practice) conditions mean a level of development according to good manufacturing practices: protocol/reagent/substance tested using reagents, tools and technical expedients that allow the production to be carried out in a pharmaceutical workshop authorized for the production of a potential clinical batch (e.g. within a clinical protocol).

The highly selected subpopulation thus isolated in a clinically significant number can be discriminated through positive or negative selection (or both) of surface markers, through detection with specific antibodies.

The therapeutic sector of interest of the population of cells that can be obtained with the isolation method of the present invention is, by way of non-limiting example, heart diseases that currently have no effective treatment.

Through the method for the isolation of the population of progenitor cells according to the present invention it is therefore possible to extend the therapeutic application of cardiac progenitors to a large number of subjects. In particular, the present method offers the possibility to obtain an extremely versatile cell therapy instrument for advanced heart failure (of ischemic and non-ischemic origin) and refractory ischemic cardiomyopathy. The method is further applicable in both an autologous and allogeneic cell therapy context, considering the maintained cell identity following cryo-conservation.

Therefore, the subject matter of the present invention is a method for the isolation of subpopulations of human cardiac progenitor cells (hCPCs) comprising the

following steps:

a) mincing a heart tissue sample containing a population of cardiac cells;

b) 4 times progressive digestion of the cardiac tissue with an enzymatic mixture until a cell suspension is achieved and filtration through filters of size comprised between 30 μM and 100 μM;

c) further digestion of residual cardiac tissue obtained in step b) for 16 hours with an enzymatic mixture until a cell suspension is achieved and filtration through filters of size comprised between 30 μM and 100 μM;

d) culturing the cell suspension obtained in step b) and c) in a culture medium suitable to propagate the cardiac cells;

e) preliminary expansion the cell suspension obtained in step d) in a culture medium suitable to expand the cardiac cells in the presence of an enzymatic or non enzymatic solution;

f) secondary expansion of the cardiac cells obtained in step e) in a culture medium suitable to further expand the cardiac cells in the presence of an enzymatic or non enzymatic solution;

g) isolating one or more subpopulations of cardiac cells by positive and/or negative selection by the use of monoclonal antibodies directed against one or more surface antigens expressed in the starting population of cardiac progenitor cells.

Preferably said isolating step is carried out by immunomagnetic sorting.

The sample of heart tissue of step a) may come from any region of the cardiac organ and its annexes containing progenitor cells, either from fresh or frozen tissue. In a particularly preferred embodiment, said heart tissue sample is a bioptic sample.

Preferably, said heart tissue sample contains a primary population of cardiac cells from the right auricle, left auricle, septum, apex or ventricular biopsy (see Figure 3). Even more preferably, the heart tissue sample is from the right auricle.

According to alternative embodiments of the aforementioned method, the starting heart tissue sample can come indifferently from adult subjects, children or foetuses.

The mincing step of step a) can occur in manual or automatic mode. The manual mode envisages the use of tweezers or surgical scissors in sterile conditions.

Step b) can be performed in four enzymatic digestion steps in order to obtain a viable cell suspension (4x30' digestion). Step c) allows to complete the digestion of the residual cardiac tissue obtained in step b) and maximize the number of in vitro expandable viable cells under GMP conditions. Preferably the 16-hours digestion of step b) is carried out overnight (O/N). Figure 4 shows the comparison of the cells obtained after 4x30' digestion step of the cardiac tissue employed in the methods previously used by the inventors (see references [4] and [5]) alone or in combination with the complete digestion of the cardiac tissue fragment obtained adding a further 16 hours digestion. The treatment according to the invention allows to double the cell number obtained at the end of step b) (passage P0-P1).

The enzymatic digestion of step b and c) preferably takes place with an enzymatic mixture comprising a basal medium, preferably Ham's/F12, and an enzymatic solution. Preferably, said enzymatic solution comprises a mixture of collagenases and/or proteases. Even more preferably, said mixture of collagenases and/or proteases comprises one or more enzymes selected from collagenase I, collagenase II, collagenase IV, trypsin, EDTA, Accutase™, collagenase A, Dispase™ and Liberase™ (i.e. NB4 or NB6 (GMP), Serva).

Preferably, the ideal medium for multiplying and expanding the cardiac cells in step d), e) and f)) comprises a basal medium, preferably Ham's/F12, serum, preferably 10% fetal bovine serum (FBS), other factors necessary for cell growth, specifically 2 mM L-glutathione, $5x10^{-3}$ U/ml of human erythropoietin, 10 ng/ml of basic fibroblast growth factor (bFGF or FGF2) and antibiotics, specifically penicillin up to 1000 U/ml and streptomycin up to 1000 µg/ml. This medium is indicated below as F12H. The initials F12G indicates the same medium but used in GMP conditions.

Alternatively, it is also possible to use other media for the growth of the cardiac cell populations, in particular the basal medium can be selected from the group consisting of MEM, DMEM, Medium199, DMEM/F12, IMDM, neurobasal

medium, EBM-2, α-MEM, mesencult or HCSCEM. Alternatively to bovine serum, horse serum can also be used. As further additional or alternative growth factors it is possible to use endothelial growth factor (EGF), leukemia inhibitory factor (LIF), sodium selenite, insulin, ascorbic acid, heparin, hydrocortisone, transferrin, 2-mercaptoethanol, L-glutamate, B27, transforming growth factor-beta 1, bone morphogenetic protein 2 (BMP-2) and bone morphogenetic protein 4 (BMP-4), insulin-like growth factor (IGF-1 and IGF-2), activin A, cardiotrophin-1, bovine brain extract (BBE) and thrombin. As antibiotics, gentamicin and amphotericin B can alternatively be used.

Furthermore, as an alternative to plastic for cell cultures, it is also possible to grow the cells on different plastics or on different supports, such as polylysine, fibronectin or porcine gelatin.

According to a particularly preferred embodiment, said enzymatic mixture comprises Ham's/F12 basal medium and the mixture of collagenase NB4 or NB6 Serva.

Preferably, said mixture of collagenase is present in a concentration comprised between 0.1 and 3 mg/ml.

Thanks to the complete enzymatic digestion of the bioptic sample, the aforementioned method allows the extraction of cells and cell clones more quickly and efficiently with respect to known protocols.

The cell suspension double expansion steps e) and f can be performed in one or more steps maintaining the expression of the main surface markers through the use of an enzymatic or non enzymatic solution that does not damage the cell membrane. Preferably, said enzymatic or non enzymatic solution is selected from the group that consists of TrypLE™ Select, a mixture of trypsin and/or EDTA, cell dissociation buffer (GIBCO), Accutase™ or Dispase™.

Preferably, the suitable culture medium for expanding the cardiac cells is F12H as described above.

The cell population obtained from the primary culture of step c) can be cryo-conserved by maintaining the expression of the main surface markers.

It is commonly considered that non-embryonic progenitor/stem cells grow very

slowly in medium and that it is necessary to separate them from differentiated cells, to prevent undesired dilutions and/or differentiations.

Thanks to the method according to the present invention, the cardiac stem and progenitor cells grow more quickly when they are maintained in a mixed population of differentiated cells.

With this new procedural expedient which envisages a preliminary and a secondary expansion step - unlike the methods known in the background (see ref. [1], [4], [5]) - it is possible to massively expand the progenitor subpopulation of interest before the final cell isolation process (see Figure 7) and to enhance the final purity.

This additional expansion step allows to obtain at least 10 times the number of starting cells in comparison to the prior art (rif. [4], Figure 7), considering as starting material a fragmen of auricle of average weight of 141,4 ± 28.29 mg and allowing to select the population of interest also in GMP-grade conditions (see Example 4).

Another advantage due to the increasing number of starting cells before selection is that the method of the invention does not require further post-selection expansion steps of the selected cells because the obtained number is already sufficient to be employed in clinical trials of cell therapy (considering the number of cells injected in the clinical trial in this sector). This leads to another advantage in terms of "quality", because by avoiding post-selection expansion step it is possible to prevent the dilution of the selected population of interest, which, as known from the literature, is not expandable as such because the culture has the intrinsic problem of the partial differentiation of the population itself.

The isolation of the population of interest can therefore be obtained starting from a much larger number of cells (at least 10 times with respect to the state of the art), therefore isolating a significantly high number of selected progenitor cells.

The method based on the isolation of a population through the use of specific antibodies that recognize a surface antigen, makes it possible to isolate any cardiac cell subpopulation, even rare, thanks to a signal amplification system.

The proposed method is a decisively quicker method (on average 22 days) with

respect to others used for obtaining a similar cell population (see Figure 6b). In fact, the state of the art shows that an average of 66.5 days are necessary for obtaining a decisively lower quality cell product (in terms of number and purity).

Therefore, the method according to the present invention allows one or more cardiac cell subpopulations to be obtained with high purity for one or more selected surface markers, as well as a clinically significant quantity of cells.

The specific antibodies used in the isolation step g) of the method according to the invention are preferably marked monoclonal antibodies (e.g. with biotin), more preferably marked with a fluorescent molecule.

Preferably said fluorescent molecule is selected from fluorescein (FITC), allophycocyanin (APC) or another fluorescent marker suitable to be combined with a magnetic selection system. It is possible to perform direct or indirect marking of the aforementioned antibodies.

Furthermore, said antibodies can be primary or secondary (only for indirect marking).

According to a preferred embodiment, the separation according to step g) is an immunomagnetic separation and the monoclonal antibodies are coupled to magnetic beads.

Alternative possible selection systems that can be used to carry out the separation according to step g) according to the invention are Dynabeads Magnetic Separation Technology Thermo Fisher Scientific, EasySep™ Magnet STEMCELL or flow cytometry cell Sorting (i.e. FacsAria BD).

According to a preferred embodiment of the method, when the cardiac tissue sample comes from the right auricle or the septum (see subsequent Example 5 of characterization using FACS analysis) it is possible to use monoclonal antibodies optionally marked with a fluorescent marker preferably selected from biotin, FITC and APC, directed against one or more antigens selected from the group of antigens listed in the following Table 1:

Table 1

CD1a	CD1b	CD1c	CD1d	CD2
CD3	CD4	CD5	CD6	CD7
CD8a	CD9	CD10	CD11a	CD11b
CD11b activated	CD11c	CD13	CD14	CD15 (SSEA-1)
CD16	CD18	CD19	CD20	CD21
CD22	CD23	CD24	CD25	CD26
CD27	CD28	CD29	CD30	CD31
CD32	CD33	CD34	CD35	CD36
CD38	CD39	CD40	CD41	CD42b
CD43	CD44	CD45	CD45RA	CD45RB
CD45RO	CD46	CD47	CD48	CD49a
CD49c	CD49d	CD49e	CD49f	CD50 (ICAM-3)
CD51	CD51/61	CD52	CD53	CD54
CD55	CD56 (NCAM)	CD57	CD58	CD59
CD61	CD62E	CD62L	CD62P (P-Selectin)	CD63
CD64	CD66a/c/e	CD66b	CD69	CD70
CD71	CD73	CD74	CD79b	CD80
CD81	CD82	CD83	CD84	CD85a (ILT5)
CD85d (ILT4)	CD85g (ILT7)	CD85h (ILT1)	CD85j (ILT2)	CD85k (ILT3)
CD86	CD87	CD88	CD89	CD90 (Thy1)
CD93	CD94	CD95	CD96	CD97
CD99	CD100	CD101 (BB27)	CD102	CD103
CD104	CD105	CD106	CD107a (LAMP-1)	CD108
CD109	CD111	CD112 (Nectin-2)	CD114	CD115
CD116	CD117 (c-kit)	CD119 (IFN-g R alpha chain)	CD122	CD123
CD124	CD126 (IL-6 R alpha)	CD127 (IL7 R alpha)	CD129 (IL-9 R)	CD131
CD132	CD134	CD135	CD137 (4-1 BB)	CD137L (4-1 BB Ligand)
CD138	CD140a	CD140b	CD141	CD143
CD143	CD146	CD148	CD150 (SLAM)	CD152
CD154	CD155 (PVR)	CD156c (ADAM10)	CD158a/h	CD158b (KIR2DL2/L3,NKAT2)
CD158d	CD158e1 (KIR3DL1,NKB1)	CD158F	CD161	CD162
CD163	CD164	CD165	CD166	CD167a (DDR1)
CD169	CD170 (Siglec-5)	CD172a (SIRPa)	CD172b (SIRPg)	CD172g (SIRPg)
CD178 (Fas-L)	CD179a	CD179b	CD180 (RP105)	CD181 (CXCR1)
CD182 (CXCR2)	CD183	CD184 (CXCR4)	CD193 (CCR3)	CD195 (CCR5)
CD196	CD197 (CCR7)	CD200 (OX2)	CD200 R	CD201 (EPCR)
CD202b (Tie2/Tek)	CD203c (E-NPP3)	CD205 (DEC-205)	CD206 (MMR)	CD207 (Langerin)
CD209 (DC-SIGN)	CD210 (IL-10 R)	CD213a2	CD215 (IL-15 Ralpha)	CD218a (IL-18R alpha)
CD220	CD221 (IGF-1R)	CD226 (DNAM-1)	CD229 (Ly-9)	CD231 (TALLA)
CD235ab	CD243	CD244 (2B4)	CD245 (p220/240)	CD252 (OX4OL)
CD253 (Trail)	CD254	CD255 (TWEAK)	CD257 (BAFF,BLYS)	CD258 (LIGHT)
CD261 (DR4,TRAIL-R1)	CD262 (DR5,TRAIL-R2)	CD263 (DcR1,TRAIL-R3)	CD266 (Fn14,TWEAK R	CD267 (TACI)
CD268 (BAFF-R,BAFFR)	CD270 (HVEM)	CD271	CD273 (B7-DC,PD-L2)	CD274 (B7-H1,PD-L1)
CD275 (B7-H2,B7-RP1,ICOSL)	CD276	CD277	CD278 (ICOS)	CD279 (PD-1)
CD282 (TLR2)	CD284 (TLR4)	CD286 (TLR6)	CD290	CD294
CD298	CD300e (IREM-2)	CD300F	CD301	CD303
CD304	CD307	CD307d (FcRL4)	CD314 (NKG2D)	CD317
CD318 (CDCP1)	CD319 (CRACC)	CD324 (E-Cadherin)	CD325	CD326 (Ep-CAM)
CD328 (Siglec-7)	CD334 (FGFR4)	CD335 (NKp46)	CD336 (NKp44)	CD337 (NKp30)
CD338 (ABCG2)	CD340 (erbB2_HER-2)	CD344 (Frizzled-4)	CD351	CD352 (NTB-A)
CD354 (TREM-1)	CD355 (CRTAM)	CD357 (GITR)	CD360 (IL-21R)	beta2-microglobulin
BTLA	C3AR	C5L2	CCR10	CLEC12A
CLEC9A	CX3CR1	CXCR7	delta-Opioid Recepto	DLL1
DLL4	DR3 (TRAMP)	EGFR	erbB3/HER-3	FceRIalpha
FcRL6	Galectin-9	GARP (LRRC32)	HLA-A,B,C	HLA-A2
HLA-DQ	HLA-DR	HLA-E	HLA-G	IFN-g R b chain
Ig light chain k	Ig light chain omega	IgD	IgM	IL-28RA
Integrin alpha9beta1	Integrin beta5	Integrin beta7	Jagged 2	LAP
Lymphotoxin beta receptor (L	Mac-2 (Galectin-3)	MAIR-II	MICA/MICB PE	MSC(W3D5)
MSC(W5C5)	MSC(W7C6)	MSC and NPC(W4A5)	MSCA-1(MSC,W8B2)	NKp80
Notch1	Notch2	Notch3	Notch4	NPC(57D2)
Podoplanin	Pre-BCR	PSMA	Siglec-10	Siglec-8
Siglec-9	SSEA-1	SSEA-3	SSEA-4	SSEA-5
TCRg/d	TCR Vbeta13.2	TCR Vbeta23	TCR Vbeta8	TCR Vbeta9
TCR Vdelta2	TCR Vg9	TCR Valpha24-Jalpha18	TCR Valpha7.2	TCR alpha/beta
Tim-1	Tim-3	Tim-4	TLT-2	TRA-1-60R
TRA-1-81	TSLPR (TSLP-R)			

According to a preferred embodiment of the isolation method according to the inventionauricle the monoclonal antibodies to perform the positive and/or negative selection of the subpopulations of cardiac progenitor cells of interest according to step g) of the method according to the invention are anti-CD117 and/or anti-CD90.

Therefore, according to a preferred embodiment of the present invention refers to a subpopulation of human cardiac progenitor cells obtained through the isolation method outlined above characterized by a profile of surface antigens selected between $CD90^-$ and $CD117^+/CD90^-$, , according to whether negative selection (see Example 2) or simultaneous double positive and negative selection (see Example 3) are used. In particular, the subpopulation of human cardiac progenitor cells hCPC $CD90^-$ showed high angiogenic and cardioprotective potential (see Figures 20-21), in addition to a significant anti-inflammatory effect (see Figure 22). Also the subpopulation $CD117^+/CD90^-$ showed a significant anti-inflammatory effect (see Figure 22). The reduced collagen production revealed by the hCPC $CD90^-$ subpopulation render it a suitable candidate for transplant (see Figure 23).

Therefore, a further object of the present invention is a subpopulation of human cardiac progenitor cells $CD90^-$ and $CD117^+/CD90^-$, selected by the isolation method outlined above, for use in the medical field. The subpopulations obtainable through the isolation method of the present invention can be used alone or also in combination with each other or with other human cardiac progenitor cells subpopulations (e.g. $CD117^+$, $CD117^-$, $CD90^+$, $CD117^+/CD90^+$, $CD117^-/CD90^-$ and $CD117^-/CD90^+$ subpopulations).

The invention further relates to the use of human cardiac cell subpopulations obtained through the isolation method outlined above in cardiovascular cell therapy or in the cardiac cell and/or tissue transplantation field.

The diseases that can benefit from the present invention include but are not limited to acute and chronic heart disease, diseases of ischemic or non-ischemic origin, myocardial diseases or lesions, cardiovascular diseases of genetic origin, congenital heart defects, valvular heart disease, arrhythmia including malignant forms, congestive and non-congestive heart failure, subendocardial fibrosis, left or

right ventricular hypertrophy, dilation of the left ventricle, myocardial acute infarction, restenosis, myocarditis, idiopathic dilated cardiomyopathy, chronic ischemic cardiomyopathy, dystrophyc cardiomyopathy (e.g. Duchenne or Becker dystrophy), pericardial diseases and/or disorders, angina pectoris including refractory forms, diseases or disorders of the blood vessels (atherosclerosis, aneurysms, arterial inflammation, all diseases of the arteries, arterioles and capillaries and related structures, including narrowing of the peripheral arteries and critical ischemia of the lower limbs), hypertension, autoimmune diseases.

Heart protection, immunoregulatory capacity and the cardiac regeneration potential of the cells selected with the aforementioned method according to the invention, make it a suitable instrument for the treatment of various diseases also including the wound repair processes or regeneration therapy.

The present invention will now be described, for non-limiting illustrative purposes, according to a preferred embodiment thereof, with particular reference to the attached figures, wherein:

- Figure 1, panels A) and B) show a view of the fragment of right auricle just harvested during aorta-coronary bypass surgery, before cleaning, weighing and mincing. Panels C) and D) show the two faces of the fragment after a longitudinal cut. Panel E) shows the fragments obtained after manual mincing. Panel F) illustrates a diagram showing the mean weight (expressed in mg of tissue) with the related standard deviation of the collected and processed auricle.

- Figure 2 shows two histograms of digestion of the cardiac tissue through the use of enzymatic solution according to research or GMP standards. The use of research and GMP enzymatic solution allows an equivalent number of cells to be obtained at the end of step 0 (P0-P1).

- Figure 3 shows two diagrams that reflect the examples of isolation of the cell population containing the cardiac progenitors from different sources of cardiac tissue (auricle and septum). From different regions of the heart a comparable number of cells can be obtained at the end of the first step (P1-P2) in the same conditions.

- Figure 4 shows a graph that demonstrates how the complete digestion of the

fragment of cardiac tissue obtained by adding an O/N digestion of the tissue not yet digested after the first digestion step (4x30') doubles the number of cells that are obtained at the end of step 1 (P1-P2).

- Figure 5 shows the images obtained under the optical microscope of a primary culture in vitro 5 days after being cultured. Panel B) shows a clone, presumably generated from a single progenitor cell, very frequent in this type of culture.

- Figure 6, panel A) shows a diagram that illustrates the average time necessary for each step in the culture medium, comparing fresh and frozen samples, from which it appears clear how the technique can be reproduced in both cases without statistically significant variations to the growing times. Panel B) shows the sum of the days from the primary culture to the selection of the population of interest (P3) both in fresh and frozen processed samples, subdivided by steps. The possibility of freezing was also proved in relation to the maintenance of the phenotype of interest. Panel C) shows the diagrams that demonstrate the expression of the antigen c-kit (CD117) before and after freezing the non-selected population.

- Figure 7 shows a graph illustrating the number of cells obtained from the complete expansion of four samples of cardiac tissue (right auricle) until the end of step 2 (P2-P3).

- Figure 8 shows the cytofluorimetric analyses reporting the percentage of progenitor cells of interest during the different steps. Panel A) shows a graph that illustrates the maintenance of the percentage of c-kit (CD117) in the population not selected during steps P2 and P3. Panel B) comprises the diagrams that show the expression of the antigen c-kit in the culture steps P2 and P3.

- Figure 9 shows an example of positive selection for the antigen c-kit. The diagrams provided show said population identified by physical parameters, while the expression of the antigen of interest is highlighted using a fluorescent antibody. Panel A) comprises the diagrams that show the analysed population identified by physical parameters, while the expression of the antigen of interest is highlighted using a fluorescent antibody. Panel B) presents an image representing the marked population only with the isotype conjugated with APC before selection used as a negative control of the non-specific marking (inside gate R2);

panel C) illustrates the expression of the marker of the population before selection (inside gate R3) and panel D) shows the selection of the positive population after selection (inside gate R3).

- Figure 10 shows an example of negative selection for the antigen CD90. The diagrams provided show the analysed population identified by physical parameters (panel A), while the expression of the antigen of interest is highlighted using a biotinylated antibody coupled to a secondary fluorescent antibody. Panel B) shows an image representing the population marked only with the secondary antibody (anti-Biotin marked with FITC) before selection (inside gate H). Panel C) shows the expression of the marker in the population before selection (inside gate H) and panel D) shows the selection of the negative population after selection (inside gate H).

- Figure 11 shows an example of the double selection of a negative population for one marker (CD90) and positive for another marker (c-kit). The diagrams provided show the population analysed identified by physical parameters in panel A); panel B) presents an image representing the marked population only with the isotype conjugated with FITC before selection and APC (panel C) used as a negative control of the non-specific marking. Panels D) and E) show the expression of the antigens of interest CD90 conjugated to fluorochrome FITC (panel D) and c-kit conjugated to fluorochrome APC (panel E). In particular, panel D) shows the population of interest for negative selection (inside gate «-»); panel E) shows the population of interest for positive selection (inside gate «+») and panel F) shows the selection of the population identified by the expression of two markers after selection (inside gate «L-+»).

- Figure 12 illustrates a diagram of the isolation method according to the invention that also allows the population to be selected that expresses the antigen of interest (c-kit) characterized by an intermediate or low mean fluorescence intensity (panel A). Panel B) shows a table comparing the number of cells, the purity and the cell viability, of the cell population obtained with the method according to the invention with respect to the prior art [4].

- Figure 13, panel A) shows a diagram of the process of seeding cells in a plate

equipped with Transwell® culture inserts that allow the simultaneous growing of different cell types that are physically separated but share the same culture medium for possible growing after selection. In the case exemplified the selected cells are plated inside the Transwell® inserts in the upper chamber and the supported cells are plated in the lower chamber. Panel B) shows some examples of cultures of the selected cells. When placed back in culture the cells are viable and start growing again, also forming clones (a peculiar characteristic of progenitor/stem cells).

- Figure 14 shows graphs (immunosorting) that demonstrate the maintenance of the antigen of interest CD90 just selected (in this example negative selection visible inside gate I) and also after subculture of the selected cells.

- Figure 15 shows an example of maintenance of the marker used for selection straight after selection and after freezing and defrosting. In the example, the positive selection was performed for the surface marker CD90 which is expressed by 98.67% of the population straight after isolation and 97.32% after the defrosting of the cells.

- Figure 16 is a diagram that shows a population of cardiac progenitors after selection characterized by positivity for c-kit in GMP conditions evaluated with both of the possible markings, using as negative control the isotype APC (full histogram), the APC human antibody anti-c-kit (grey broken line) and the antibody anti-biotin marked with APC (light continuous line).

- Figure 17 shows a table of the surface antigens evaluated in the sample of cardiac tissue of the auricle and septum.

- Figure 18 shows endothelial differentiation of hCPC subpopulations assessed by Cultrex assay. Results display tubular structures formation in hCPC-ns, hCPC-$CD117^+$, hCPC-$CD90^+$ and hCPC-$CD90^-$ on Cultrex membrane. Panel A) Bar graphs show the quantification of tubular-like structures per microscopic field; Panel B) Bar graphs show the number of branching points between tubular-like structures. In these conditions hCPC-$CD90^-$ subpopulation produce a significant more tubular-like structures in 4 hours compared to the other subpopulations considered (hCPC-ns); (n = 4) * $p < 0.05$, ** $p < 0.05$, *** $p < 0.001$.

- Figure 19 shows endothelial differentiation of hCPC subpopulations evaluated by FACS analysis. Bar graph shows the comparison between VE-cadherin/CD144$^+$, CD146$^+$ and VEGFR-2/KDR$^+$ cells in hCPC-CD117$^+$ and hCPCCD90$^-$ cells after culturing into pro-angiogenic EGM-2 medium. In these conditions hCPC-CD90- cells shown a significant higher expression of endothelial markers CD144 and KDR. (n=4) *p<0.05.

- Figure 20 shows the expression profiles of growth factors and cytokines in hCPC subpopulations. Bar graphs show an increase in cytokines and growth factors release in different hCPC subpopulations (hCPC-CD117$^+$, hCPC-CD90$^-$ and hCPC-CD90$^+$) compared to the unselected population (hCPC-ns). From the comparison hCPC-CD90$^-$ subpopulation shows an increased secretion profile for many factors, reaching the statistical significance for IL-6 and IL-8 compared to hCPC-CD117$^+$ subpopulation (n = 3) * p < 0.05.

- Figure 21 shows cardioprotective potential of hCPC subpopulations. Bar graphs show the release of cardiac troponin isoform I (cTnI) in cardiomyocytes derived from iPS (CM-d-hiPSCs) from patients with dystrophic cardiomyopathy (Duchenne and Becker). It is evident that hCPC-CD90$^-$ subpopulation shows the highest and significant cardioprotective effect on both CM-d-hiPSCs populations, inducing the lowest troponin release. (n = 6) * p <0.05, ** p <0.05, *** p <0.001.

- Figure 22 shows the anti-inflammatory effect of hCPC subpopulations. Bar graphs show the release of TNF-α in cardiomyocytes derived from iPS (CM-d-hiPSCs) of patients with dystrophic cardiomyopathy. Panel A): TNF-α release in CM-d-hiPSCs supernatant (from Duchenne patients) co-cultured in the presence of hCPC-ns, hCPC-CD117$^+$, hCPC-CD90$^+$ and hCPC-CD90$^-$ after 3 days from the beginning of the co-culture; under these conditions the hCPC-CD90$^-$ subpopulation shows a significant anti-inflammatory effect, reducing the TNF-α release (n = 4). Panel B: TNF-α release in CM-d-hiPSCs supernatant (from Becker patients) co-cultured with hCPC-ns, hCPC-CD117$^+$, hCPC-CD90$^+$ and hCPCCD90$^-$ 7 days after the beginning of the co-culture; under these conditions hCPC-CD90$^-$ and hCPC-CD117$^+$/CD90$^-$ subpopulations shows significant anti-inflammatory effect (n = 6). * p <0.05, ** p <0.05, *** p <0.001.

- Figure 23 shows myofibroblast differentiation of hCPC subpopulations evaluated by collagen production *in vitro* after TGF-β1 treatment for 5 days. The comparison shows that hCPC-CD90$^-$ subpopulation has the lowest ability to produce collagen compared to other populations considered (hCPC-ns, hCPC-CD117$^+$ and hCPC-CD90$^+$); (n = 3) * p <0.

EXAMPLE 1: *Method for the isolation of a population of human cardiac progenitor cells by positive selection*

MATERIALS AND METHODS

Primary culture

The sample of human auricle with a weight comprised between 36.8 and 631.8 mg (see Figure 1) is harvested in the operating theatre and immediately transferred into a sterile container containing at least one sterile solution to prevent the dehydration thereof (phosphate-buffered saline, PBS or physiological solution) and preferably a solution that also maintains the viability of the tissue (e.g. any culture medium)

Alternatively, it is also possible to use another solution containing bovine serum albumin (BSA) or human serum albumin (HSA) or fetal bovine serum (FBS). From that moment the sample can be kept at controlled temperature (+4°C) and processed within 48 hours or frozen in liquid nitrogen in a solution containing at least FBS and DMSO as the freezing does not have any effect on whether a culture is obtained (see Figure 6a-b). For processing, after appropriate preparation with particular reference to the epicardium removal process, the fragment is weighed and minced using microsurgical tweezers and scissors into fragments of about 1-2 mm^3 and transferred into a tube together with the digestion solution (generally 1.7 ml of solution for every 100 mg of starting tissue). The digestion solution comprises basal culture medium, preferably Ham's/F12 containing collagenase NB4 (SERVA) at the concentration of 3 mg/ml (see Figure 2).

Digestion of the fragment

1. The tube(s) containing the fragments of tissue is/are transferred into a rotating oscillator that allows the movement of the fragments inside the tube at the temperature of 37°C until the solution becomes cloudy due

to the effective digestion of the fragments themselves (generally 30-40 minutes or up to 4 hours).

2. The tube(s) is/are recovered and the fragments that have not yet been digested can be deposited on the bottom of the tube; the "digested" solution (containing the cells) is recovered and transferred into a tube which, after the addition of PBS, will be centrifuged to allow the cells to be deposited on the bottom (it is centrifuged at 4°C at 400 g for 10 minutes or 800 g for 5 minutes).
3. The supernatant is removed, the cells are re-suspended in complete medium and the tube containing the cells in ice is transferred, pending subsequent digestions. In the specific case, the complete medium is comprised of: Ham's/F12 containing 10% of fetal bovine serum (FBS), 2 mM L-glutathione and $5x10^{-3}$ U/ml of human erythropoietin, 10 ng/mL of basic fibroblast growth factor (bFGF or FGF2) and antibiotics (penicillin up to 1000 U/ml and streptomycin up to 1000 ug/ml): F12H.

In the meantime a new digestion solution is added to the tube containing the fragments of tissue not yet digested repeating the previous steps 1-3 for a total of four times.

The four digestions conserved in ice are collected and transferred into a syringe connected to a syringe filter, preferably of 70 μM to be filtered in a new tube. The 4 tubes with complete medium are washed and the washing liquid is also transferred into the same syringe for filtering.

In the specific case, Ham's/F12 medium is used containing 10% of fetal bovine serum (FBS), 2 mM L-glutathione and $5x10^{-3}$ U/ml of human erythropoietin, 10 ng/mL of basic fibroblast growth factor (bFGF or FGF2) and antibiotics (penicillin up to 1000 U/ml and streptomycin up to 1000 ug/ml): F12H.

The filtered solution is plated in a sterile capsule such as a Petri dish (generally one 100 mm diameter plate for every 100 mg of starting medium) (Day 1).

To allow the total digestion of the fragments still in the tube(s) a new digestion solution is added, preferably at a concentration of up to 0.3 mg/ml.

The tube(s) containing the fragments of tissue is/are transferred into a rotary

oscillator that allows the movement of the fragments inside the tubes at the temperature of 37°C for a whole night (about 16 hours).

The digested solution is filtered using a nylon mesh filter, preferably of 70 μM, and the filtrate is collected. The same filter is washed with PBS and the filtered solution is centrifuged to allow the cells to settle on the bottom.

The supernatant is removed, the cells are re-suspended in complete medium F12H and plated in a sterile capsule such as a Petri dish (generally a 100 mm diameter plate for every 100 mg of starting medium). The tube can be washed with complete medium F12H and the washing liquid transferred into the same Petri dish, for recovering any cells still in the tube (Day 2).

48 hours after being cultured (Day 3 for 4x30' digestions and Day 4 for the fragments digested all night) the culture medium containing the detritus, the dead cells or that have not adhered, are completely removed, the plate is washed with PBS and new fresh medium F12H is added (Figure 4).

The medium is changed, preferably every 2 days.

The growth of the cells is checked under the microscope and when about 70% confluence is reached or when the clones present become too confluent, the amplification of the cells is performed (Figure 5).

<u>Amplification of the non-selected population</u>

4. The culture medium is removed, the surface of the Petri capsule where the cells are plated is washed with PBS and the solution is added which can detach the cells from the plastic that is to be incubated at a controlled temperature of 37°C for the necessary time to obtain a cell suspension. For this process it is possible to use an enzyme solution (trypsin) or a non-enzyme solution (Tryple™ Select, EDTA, Cell dissociation buffer or Cell Stripper™).
5. The reaction is interrupted by the addition of a solution containing FBS (if the enzyme solution has been used) or PBS (if the non-enzyme solution has been used). The solution containing the cells is centrifuged in a tube.
6. The cells are counted and re-plated in F12H medium in a 1:10 dilution

or preferably at the concentration of 1200-1500 cells/cm^2. The medium is changed, preferably every 2 days.

The growth of the cells is checked under the microscope and when 70% confluence is reached, further amplification of the cells is performed (by repeating steps 4 to 6). In fact, the further step does not compromise the frequency of the antigen of interest which identifies the population of interest that will be selected subsequently (Figure 8). Alternatively, it is also possible to cryo-conserve the cell population obtained and complete the isolation at a later date as cryo-conservation does not compromise the frequency of the antigen of interest that identifies the population of interest that will be selected (see Figure 6c).

Isolation of the population of interest by positive selection

The population to be isolated is detached from the Petri capsule using the non-enzymatic method (see section "Amplification of the non-selected population"), the cells are counted and re-suspended in the preferably cold isolation buffer (Wash Buffer, WB); in the specific case, PBS is used containing ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA or HSA): the cells were re-suspended at the concentration of 100 µl every 1x10^6 cells.

A part of the cells (100,000 cells) are used for fluorescence-activated cell sorting (FACS) analysis before selection.

The cells were marked with IgG isotype immunoglobulin conjugated with the same fluorescent molecule bonded to the antibody that recognises the population of interest (in this example APC), and incubated for 15 minutes in the dark at ambient temperature or with the anti-biotin antibody marked with APC and incubated for 15 minutes in the dark at +4°C. After marking the cells were washed with WB and analysed with FACS (tube identified as isotype) (see Figure 9a-b).

The remaining cells were marked with the antibody for selection; the cells were marked with anti-human CD117 antibody marked with biotin at the concentration of 2 µg every 1x10^6 cells for 20 minutes at +4°C in agitation.

After marking the cells were washed with WB preferably cold and centrifuged. The supernatant was removed and the cells were re-suspended in WB at the

concentration of 160 µL every $1x10^6$ cells.

A part of the cells (100,000 cells) are used for fluorescence-activated cell sorting (FACS) analysis before selection. The cells were marked with anti-biotin antibody marked with APC, and incubated for 15 minutes in the dark at +4°C. After marking the cells were washed with WB and analysed through FACS (tube identified as Marked) (Figure 9c).

The remaining cells are marked with the secondary antibody; microspheres were used with anti-biotin antibodies at the concentration of 40 µl every $1x10^7$ cells, marking the cells for 15-20 minutes at +4°C in agitation.

After marking the cells were washed with WB preferably cold and centrifuged. The supernatant was removed and the cells were re-suspended in cold WB; the cells were re-suspended in a volume of 500 µl up to $1.5x10^6$ cells or 1 ml up to $15x10^7$ cells for subsequent magnetic separation.

Magnetic separation

The magnetic separation can be performed using, by way of example, the columns and the magnet made by the company Miltenyi. The column is activated using WB preferably cold (500 µl for the MS column and 1 ml for the LS column). The negative population is collected in a tube (NEG). The cells are transferred into the magnetic columns (up to $1.5x10^6$ cells in an MS column or $15\text{-}30x10^7$ cells in an LS column). The columns are washed 3 times adding WB preferably cold (500 µl for the MS column and 1 ml for the LS column). The positive population is recovered by squeezing the contents of the column into a tube after detaching it from the magnet and WB is added preferably cold (1 ml for the MS column and 2 ml for the LS column) (POS). To increase the selection purity, the positive cells selected (POS) can be passed into a new MS or LS magnetic column based on the expected number. To increase the number of positive cells selected it is possible to pass the cells of the NEG tube into a new MS or LS magnetic column based on the starting number. The positive (POS) and negative (NEG) cells were centrifuged, re-suspended in F12H medium and counted.

Also in this case a part of the cells (100,000 cells) is used for fluorescence-activated cell sorting (FACS) analysis (Figure 9d) after selection in order to verify

the purity thereof. The cells were marked with an anti-biotin antibody marked with APC, and incubated for 15 minutes in the dark at +4°C (Miltenyi). After marking the cells were washed with WB and analysed through FACS.

After selection the cells can be frozen or plated in particular Transwell® plates equipped with culture inserts that allow the simultaneous culture of different types of cells that are physically separated but share the same culture medium (Figure 13). In particular, the positive cells were plated in F12H medium on the bottom of the culture plate at a concentration comprised between 4,000-20,000 cells/cm^2 and the negative cells on the Transwell® insert at a concentration comprised between 3,000-10,000 cells/cm^2 or vice versa.

EXAMPLE 2: *Method for the isolation of a population of human cardiac progenitor cells by negative selection*

The digestion steps of the fragment, primary culture and expansion before the selection are the same as in Example 1.

Isolation of the population of interest by negative selection

The population to be isolated is detached from the Petri dish using the non-enzymatic method (see section "Amplification of the non-selected population"), the cells are counted and re-suspended in the cold isolation buffer (WB); the cells were re-suspended at the concentration of 100 µl every $1x10^7$ cells.

A part of the cells (100,000) is used for fluorescence-activated cell sorting (FACS) analysis before negative selection. The cells were marked with immunoglobulin (isotype IgG) conjugated with the same fluorescent molecule bonded to the antibody that recognizes the population of interest (in this example FITC, BD), 1 µl of antibody and incubated for 15 minutes in the dark at ambient temperature or with the anti-biotin FITC (Miltenyi) antibody, 10 µl of antibody and incubated for 15 minutes in the dark at +4°C.

After marking the cells were washed with WB and analysed through FACS (Isotype identified tube, see Figure 10a-b).

The remaining cells were marked with the antibody for selection; the cells were marked with anti-human CD90 antibody conjugated with biotin (Miltenyi) at the concentration of 10 µl every $1x10^7$ cells for 10 minutes at +4°C or in ice.

After marking the cells were washed with cold WB and centrifuged. The supernatant was removed and the cells were re-suspended in WB at the concentration of 160 µl every $1x10^6$ cells.

A part of the cells (100,000) is used for fluorescence-activated cell sorting (FACS) analysis before selection. The cells were marked with anti-biotin antibody marked with FITC (Miltenyi), 10 µl of antibody and incubated for 15 minutes in the dark at +4°C. After marking the cells were washed with WB and analysed through FACS (tube identified as Marked, Figure 10c).

The remaining cells are marked with the secondary antibody; microspheres were used with anti-biotin antibodies (Miltenyi) at the concentration of 40 µl every $1x10^7$ cells, marking the cells for 15-20 minutes at +4°C in agitation.

After marking the cells were washed with cold WB and centrifuged. The supernatant was removed and the cells were re-suspended in cold WB; the cells were re-suspended at the concentration of 500 µl up to $30x10^7$ cells for subsequent magnetic separation.

Magnetic separation

The magnetic separation can be performed using the columns and the magnet made by the company Miltenyi. The column is activated using cold WB (2 ml for the LD column). The cells were transferred into the magnetic columns (up to $30x10^7$ cells in an LD column). The columns were washed twice adding cold WB each time (1 ml for the LD column). The negative population is collected in a tube (NEG). To increase the selection purity, the negative cells selected (NEG) were passed into a new MS or LS magnetic column based on the expected number. In this case the negative population was recovered after passing the cells into a preactivated column (with cold WB) without any subsequent washing of the column itself. If the positive population is also to be collected, it is recovered by squeezing the contents of the column into a 15 ml tube after detaching it from the magnet and adding cold WB (POS). To increase the selection purity, the positive cells selected (POS) were passed into a new MS or LS magnetic column based on the expected number. The positive (POS) and negative (NEG) cells were centrifuged, re-suspended in F12H medium and counted.

A part of the cells (100,000 cells) is used for fluorescence-activated cell sorting (FACS) analysis after selection in order to verify the purity thereof. The cells were marked with anti-biotin antibody FITC (Miltenyi), 10 µl of antibody and incubated for 15 minutes in the dark at +4°C. After marking the cells were washed with WB and analysed through FACS (Figure 10d).

After selection the cells can be frozen (see Figure 15 for an example with positive selection) or plated at a concentration comprised between 3000-5000 cells/cm^2, considering the fact that their selected phenotype does not change (Figure 14).

EXAMPLE 3: *Method for the isolation of a population of human cardiac progenitor cells by combined positive and negative selection*

The digestion steps of the fragment, primary culture and expansion before the selection are the same as in Example 1.

Isolation of the population of interest by combined positive and negative selection

The population to be isolated is detached from the Petri dish using the non-enzymatic method (see section "Amplification of the non-selected population"), the cells are counted and re-suspended in the cold isolation buffer (WB); the cells were not re-suspended at the concentration of 100 µl every $1x10^7$ cells.

A part of the cells (400,000) is used for fluorescence-activated cell sorting (FACS) analysis before selection (Figure 11A-E). Subsequently, the cells were divided into four different tubes.

The first tube (identified as Isotype FITC) was marked with the immunoglobulins (isotype IgG) conjugated with the same fluorescent molecule bonded to the antibody that recognizes the population of interest (in this example FITC, BD), 1 µl of antibody and incubated for 15 minutes in the dark at ambient temperature, the second tube (identified as Isotype APC, see Figure 11C) was marked with the immunoglobulins conjugated with the same fluorescent molecule bonded to the antibody that recognises the population of interest (in this example APC, BD), the third and fourth tube (identified respectively as CD90 FITC and c-kit APC, see Figures 11D, E) were marked with the specific respective antibodies (CD90 FITC and c-kit APC, BD) up to 5 µl of antibody and incubated for 15 minutes in the dark at ambient temperature. After marking the cells were washed with WB and

analysed through FACS.

The remaining cells were marked with the first antibody for selection (negative selection); the cells were marked with anti-human CD90 antibody conjugated to biotin (Miltenyi) at the concentration of 10 µL every $1x10^7$ cells for 10 minutes at +4°C or in ice.

After marking the cells were washed with cold WB and centrifuged. The supernatant was removed and the cells were re-suspended in WB at the concentration of 160 µl every $1x10^6$ cells.

The cells are marked with the secondary antibody; microspheres were used with anti-biotin antibodies (Miltenyi) at the concentration of 40 µl every $1x10^7$ cells, marking the cells for 15-20 minutes at +4°C in agitation. After marking the cells were washed with cold WB and centrifuged. The supernatant was removed and the cells were re-suspended in cold WB; the cells were re-suspended at the concentration of 500 µl up to $30x10^7$ cells for subsequent magnetic separation.

Negative magnetic separation

The magnetic separation can be performed using the columns and the magnet made by the company Miltenyi. The column is activated using cold WB (2 ml for the LD column). The cells are transferred into the magnetic columns (up to $30x10^7$ cells in an LD column). The columns were washed twice adding cold WB each time (1 ml for the LD column). The negative population is collected in a tube (NEG). To increase the selection purity, the negative cells selected (POS) were passed into a new MS or LS magnetic column based on the expected number. In this case the negative population was recovered after passing the cells into a pre-activated column (with cold WB) without any subsequent washing of the column itself. If the positive population is also to be collected, it is recovered by squeezing the contents of the column into a tube after detaching it from the magnet and adding cold WB (POS). To increase the selection purity, the positive cells selected (POS) can be passed into a new MS or LS magnetic column based on the expected number.

The negative cells (NEG) were centrifuged, re-suspended in cold WB and counted; the cells were not re-suspended at the concentration of 100 µl every

$1x10^6$ cells.

The cells were marked with the antibody for selection (positive selection); the cells were marked with anti-human CD117 antibody conjugated to biotin (Biolegend) at the concentration of 2 µg every $1x10^6$ cells for 20 minutes at +4°C in agitation.

After marking the cells were washed with cold WB and centrifuged. The supernatant was removed and the cells were re-suspended in WB at the concentration of 160 µl every $1x10^6$ cells to be marked with the secondary antibody; microspheres were used with anti-biotin antibodies (Miltenyi) at the concentration of 40 µl every $1x10^7$ cells, marking the cells for 15-20 minutes at +4°C under agitation.

After marking the cells were washed with cold WB and centrifuged. The supernatant was removed and the cells were re-suspended in cold WB; the cells were re-suspended at the concentration of 500 µl up to $1.5x10^6$ cells or at the concentration of 1 ml up to $15x10^7$ cells for subsequent magnetic separation.

Positive magnetic separation

The magnetic separation can be performed using the columns and the magnet made by the company Miltenyi. The column is activated using cold WB (500 µl for the MS column and 1 ml for the LS column). The negative population is collected in a tube (NEG-NEG). The cells were transferred into the magnetic columns (up to $1.5x10^6$ cells in an MS column or up to $30x10^7$ cells in an LS column). The columns were washed 3 times adding cold WB each time (500 µl for the MS column and 1 ml for the LS column). The positive population was recovered by squeezing the contents of the column into a tube after detaching it from the magnet and adding cold WB (1 ml for the MS column and 2 ml for the LS column) (POS). To increase the selection purity, the positive cells selected (NEG-POS) were passed into a new MS or LS magnetic column based on the expected number. To increase the number of positive cells selected the cells of the NEG-NEG tube were passed into a new MS or LS magnetic column based on the starting number. The positive (NEG-POS) and negative (NEG-NEG) cells were centrifuged, re-suspended in F12H medium and counted.

A part of the cells (200,000 cells) was used for the fluorescence-activated cell sorting (FACS) analysis after selection in order to verify the purity thereof (see Figure 11F). The cells were marked with mouse antibodies marked with APC anti-human CD117 (BD, up to 5 µl) and with antibodies marked with FITC anti-human CD90 (BD, 1 µl).

After selection the cells can be frozen or plated in particular Transwell® plates equipped with culture inserts that allow the simultaneous culture of different types of cells that are physically separated but share the same culture medium. In particular, the double selected cells (positivity for one marker and negativity for another) were plated in F12H medium on the bottom of the culture plate at a concentration comprised between 4000-20,000 cells/cm^2 and the cells of the same patient on the Transwell® insert at a concentration comprised between 3000-10,000 cells/cm^2 or vice versa (Figure 13).

EXAMPLE 4: *Implementation of the method of isolating cardiac progenitor cells in GMP conditions*

MATERIALS AND METHODS

Primary culture

The sample of human auricle (with a weight comprised between 36.8 and 631.8 mg) is collected in the operating theatre and immediately transferred into a sterile container containing a sterile solution to prevent the dehydration thereof (phosphate-buffered saline, PBS, physiological solution) and preferably a solution that also maintains the viability of the tissue (i.e. any culture medium).

Alternatively, it is also possible to use another solution containing bovine serum albumin (BSA) or fetal bovine serum (FBS).

From this moment the sample can be kept at controlled temperature (+4°C) and preferably processed within 48 hours or frozen in liquid nitrogen in a solution suitable for the cryopreservative. For processing, the fragment is then minced mechanically. If the mincing envisages prior cleaning of the auricle of the epicardium that covers it, the fragment is cleaned before weighing, otherwise for protocols that do not require cleaning of the fragment, it is weighed with any epicardium still covering it.

For mechanical mincing, different equipment can be used that make the passage automatable. For example, the Medimachine (BD) and the GentleMACS (Miltenyi) machines were used.

Digestion of the fragment

The minced tissues is then digested using collagenase NB-6 (GMP grade) adapting the method already developed for the basic study (Figure 2):

1. The tube(s) containing the fragments of tissue is/are transferred into a rotating oscillator that allows the movement of the fragments inside the tube at the temperature of 37°C until the solution becomes cloudy due to the effective digestion of the fragments themselves (generally 30-40 minutes or up to 4 hours).
2. The tube(s) is/are recovered and the fragments that have not yet been digested can be deposited on the bottom of the tube; the "digested" solution (containing the cells) is recovered and transferred into a tube which, after the addition of PBS, will be centrifuged at 4°C to allow the cells to settle on the bottom.
3. The supernatant is removed and the cells are re-suspended in complete medium. In the specific case, Ham's/F12 medium is used containing 10% of GMP grade fetal bovine serum (FBS), 2 mM L-Glutathione and $5x10^{-3}$ U/ml of human erythropoietin, 10 ng/mL of basic fibroblast growth factor (bFGF or FGF2) and antibiotics (penicillin up to 1000 U/ml and streptomycin up to 1000 ug/ml): F12G and the tube containing the cells in ice is transferred, pending subsequent digestions.

In the meantime a new digestion solution is added to the tube containing the fragments of tissue not yet digested repeating the previous steps 1 to 3 for a total of 4 times.

The digestions conserved in ice are collected and transferred into a syringe connected to a syringe filter, preferably of 70 µM to be filtered in a new tube. The tubes with complete medium F12G are washed and the washing liquid is also transferred into the same syringe for filtering.

The filtered solution is plated in a sterile capsule such as a Petri dish (generally

one 100 mm diameter plate for every 100 mg of starting medium) (Day 1).

To allow the total digestion of the fragments still in the tube(s) a new digestion solution is added, preferably at a concentration of up to 0.3 mg/ml.

The tube(s) containing the fragments of tissue is/are transferred into a rotary oscillator that allows the movement of the fragments inside the tube at a temperature of about 37°C for a whole night (about 16 hours).

The digested solution is filtered using a nylon mesh filter, preferably of 70 μM, and the filtrate is collected. The same filter is washed with PBS and the filtered solution is centrifuged to allow the cells to settle on the bottom.

The supernatant is removed, the cells are re-suspended in complete medium F12G and plated in a sterile capsule such as a Petri dish (generally a 100 mm diameter plate for every 100 mg of starting medium). The tube can be washed with complete medium F12G and the washing liquid transferred into the same Petri dish, for recovering any cells still in the tube (Day 2).

48 hours after being cultured (Day 3 for 4x30' digestions and Day 4 for the fragments digested all night) the culture medium containing the detritus, the dead cells or that have not adhered, are completely removed, the plate is washed with PBS and new fresh medium F12G is added (Figure 4).

The medium is changed, preferably every 2 days.

The growth of the cells is checked under the microscope and when about 70% confluence is reached or when the clones present become too confluent, the amplification of the cells is performed (Figure 5).

<u>Amplification of the non-selected population</u>

4. The culture medium is removed and the surface of the Petri capsule where the cells are plated with PBS is washed and the non-enzymatic solution is added that is able to detach the cells from the plastic (TrypLE™ Select, Life Technologies). The non-enzymatic solution is incubated with the cells at controlled temperature, around 37°C for the necessary time to obtain a cell suspension, from about 3 to 10 minutes.
5. The solution is inactivated by adding PBS. The solution containing the cells is centrifuged in a tube.

6. The cells are counted and re-plated in F12G medium in a 1:10 dilution or preferably at the concentration of 1200-1500 cells/cm^2. The medium is changed every two days.

The growth of the cells is counted under the microscope and when about 70% confluence is reached, further amplification of the cells is performed (see steps 4 to 6). For the extensive amplification, multi-flasks with 3 (525 cm^2) and 5 (875 cm^2) layers were used, such as cell-stacks with 5 layers (3180 cm^2).

Isolation of the population of interest by positive selection

The population to be isolated is detached from the multi-flask/cell-stack using the non-enzymatic method (see section "Amplification of the non-selected population"), the cells are counted and re-suspended in the isolation buffer (WB) preferably cold; in the specific case, PBS is used containing EDTA and HSA: the cells were re-suspended at the concentration of 100 µl every $1x10^6$ cells.

A part of the cells (100,000) is used for fluorescence-activated cell sorting (FACS) analysis before selection. The cells were marked with immunoglobulin (IgG isotype) conjugated with the same fluorescent molecule bonded to the antibody that recognises the population of interest (in this example APC), and incubated for 15 minutes in the dark at ambient temperature or with the anti-biotin antibodies marked with APC (Miltenyi), incubated for 15 minutes in the dark at +4°C. After marking the cells were washed with WB and analysed with FACS (tube identified as Isotype).

The remaining cells were marked with the antibody for selection; the cells were marked with anti-human CD117 antibody marked with biotin (Biolegend) at the concentration of 2 µg every $1x10^6$ cells for about 20 minutes at +4°C in agitation. After marking the cells were washed with cold WB and centrifuged. The supernatant was removed and the cells were re-suspended in WB at the concentration of 160 µl every $1x10^6$ cells.

A part of the cells (100,000) are used for fluorescence-activated cell sorting (FACS) analysis before selection. The cells were marked with anti-biotin antibody marked with APC (Miltenyi), 10 µl of antibody and incubated for 15 minutes in the dark at +4°C. After marking the cells were washed with WB and analysed

through FACS (tube identified as Marked).

The remaining cells are marked with the secondary antibody; microspheres were used with CliniMACS anti-biotin antibodies (Miltenyi) at the concentration of 40 µl every $1x10^7$ cells, marking the cells for 15-20 minutes at about +4°C in agitation.

After marking the cells were washed with cold WB and centrifuged. The supernatant was removed and the cells were re-suspended in cold WB; the cells were re-suspended in a volume of 1 ml every $1.6x10^6$ cells for subsequent magnetic separation.

Magnetic separation

The magnetic separation is performed using the instrument for magnetic selection intended for clinical use; CliniMACS Plus made by the company Miltenyi was used. Using a relevant program the positive cells were used, separated from the initial population and collected in a relevant bag, as for the negative cells.

A part of the cells (100,000) is used for fluorescence-activated cell sorting (FACS) analysis after selection in order to verify the purity thereof. The cells were marked with anti-biotin antibody marked with APC (Miltenyi), 10 µl of antibody and incubated for 15 minutes in the dark at +4°C. After marking the cells were washed with WB and analysed through FACS (Figure 16).

After selection, the cells can be frozen without losing the marker expression (see Figure 15, on CD90 positive) for which they were selected and then ready for clinical use for the cell therapy or transplantations.

EXAMPLE 5: *Identification of the surface markers selectable in two different sources of cardiac cells*

MATERIALS AND METHODS

The digestion steps of the fragment, primary culture and expansion before selection are the same as in Example 1.

In this example three different samples of auricle and three different samples of septum were used.

At the end of the third passage the three auricle were counted, joined in a single sample and marked with the fluorescent marker VioBlue450 that makes all the

cells in the V450 channel fluorescent. In the same way, the three samples from the septum were counted, joined in a single sample and marked with the fluorescent marker CFSE that makes all the cells in the FITC channel fluorescent.

The two cell populations were identified using the flow cytometer based on the marking used and subsequently the two cell populations were used to evaluate a panel of surface antigens.

The surface antigens evaluated in the sample of auricle and septum cardiac tissue are shown in the Table illustrated in Figure 17.

The marking was performed following the instructions of the manufacturer of the commercial kit LEGENDScreen™ Lyophilized Antibody Panel Human Cell Screening (PE) Kit, BioLegend.

Using an appropriate gate strategy, the two cell populations were characterized for all the surface antigens illustrated in Figure 17.

Table 2 represents the surface antigens expressed by the two different cell populations and the average expression of each antigen in the two cell sources. All the antigens expressed by at least one of the two sources used can be selected both with positive selection (see Example 1) and using negative selection (see Example 2) or, if appropriately combined also with a multiple selection of more than one marker (as shown for example in Example 3).

Table 2

	Auricle	**Septum**
Antigen	**% expression**	**% expression**
CD1a	1.5	0.97
CD1b	2.31	1.81
CD1c	1.02	0.72
CD1d	2.59	2.1
CD2	0.8	0.72
CD3	1.93	1.14
CD4	0.82	0.72
CD5	2.13	1.09
CD6	0.94	0.53
CD7	1.45	1.2

CD8a	0.09	0.21
CD9	99.85	99.04
CD10	17.99	14.45
CD11a	0.59	0.49
CD11b	2.84	1.81
CD11b activated	2.33	1.77
CD11c	1.23	0.79
CD13	98.7	99.48
CD14	5.79	6.16
CD15 (SSEA-1)	4.31	2.38
CD16	1.1	0.94
CD18	3.35	1.81
CD19	1.1	0.98
CD20	26.78	17.3
CD21	0.48	0.58
CD22	12.32	34.07
CD23	1.17	0.76
CD24	13.43	15.67
CD25	1.67	2.25
CD26	99.89	99.22
CD27	1.88	1.59
CD28	2.28	2.13
CD29	99.89	83.08
CD30	0.93	0.72
CD31	7.05	9.01
CD32	1.12	0.75
CD33	3.81	2.74
CD34	3.83	6.61
CD35	1.64	1.14
CD36	2.07	3.4
CD38	2.35	1.97
CD39	2.34	3.09
CD40	1.56	3
CD41	21.09	30.25
CD42b	1.06	0.88
CD43	6.16	8.74
CD44	99.86	90.38
CD45	0.69	1.85
CD45RA	0.53	0.64
CD45RB	0.32	0.3
CD45RO	1.3	0.76

CD46	85.69	87.43
CD47	99.55	99.75
CD48	2.65	1.69
CD49a	73.34	76.25
CD49c	99.93	99.8
CD49d	85.19	71.15
CD49e	99.92	99.92
CD49f	63.99	47.33
CD50 (ICAM-3)	0.35	0.22
CD51	99.89	99.85
CD51/61	93.35	94.24
CD52	2.93	3.16
CD53	0.37	0.57
CD54	35.28	51.1
CD55	85.71	90.12
CD56 (NCAM)	44.25	10.07
CD57	46.31	10.95
CD58	99.9	99.4
CD59	99.61	28.22
CD61	77.46	76.52
CD62E	1.59	1.21
CD62L	0.47	0.43
CD62P (P-Selectin)	1.05	0.99
CD63	99.88	99.63
CD64	4.13	2.79
CD66a/c/e	4.65	3.54
CD66b	1.54	1.51
CD69	0.67	0.75
CD70	3.63	2.28
CD71	91.68	96.73
CD73	99.86	99.54
CD74	6.67	6.57
CD79b	2.84	2.27
CD80	7.24	6.2
CD81	99.95	98.85
CD82	9.69	12.28
CD83	5.79	4.86
CD84	1.17	0.87
CD85a (ILT5)	0.79	0.83
CD85d (ILT4)	2.09	2.01
CD85g (ILT7)	0.6	0.54

CD85h (ILT1)	2.83	2.76
CD85j (ILT2)	6.9	10.53
CD85k (ILT3)	0.73	0.56
CD86	3.01	1.71
CD87	3.78	2.42
CD88	4.13	2.72
CD89	3.37	2.4
CD90 (Thy1)	57.31	31.11
CD93	2.18	1.74
CD94	1.32	0.68
CD95	99.88	99.88
CD96	1.11	0.93
CD97	86.53	85.05
CD99	99.96	98.81
CD100	3.86	2.51
CD101 (BB27)	1.39	1.24
CD102	5.62	7.61
CD103	0.14	0.28
CD104	1.92	1.35
CD105	99.67	99.79
CD106	2.47	2.52
CD107a (LAMP-1)	45.37	78.48
CD108	71.97	71.34
CD109	9.84	11.25
CD111	12.42	3.75
CD112 (Nectin-2)	99.63	99.58
CD114	4.22	2.84
CD115	6.09	5.71
CD116	3.17	2.68
CD117 (c-kit)	1.52	1.69
CD119 (IFN-g R alpha chain)	11.79	12.69
CD122	0.38	0.27
CD123	0.73	0.66
CD124	3.8	3.24
CD126 (IL-6 R alpha)	1.74	0.9
CD127 (IL7 R alpha)	2.98	2.02
CD129 (IL-9 R)	5.6	3.85
CD131	2.25	1.25
CD132	18.51	18.69
CD134	2.91	1.73
CD135	3.57	2.25

CD137 (4-1 BB)	1.57	1.23
CD137L (4-1 BB Ligand)	7.54	5.28
CD138	10.01	6.5
CD140a	22.98	30.17
CD140b	12.44	14.35
CD141	18.82	12.35
CD143	12.25	8.34
CD143	4.87	3.22
CD146	21.5	30.73
CD148	28.73	27.96
CD150 (SLAM)	0.84	0.65
CD152	1.92	1.38
CD154	1.28	0.89
CD155 (PVR)	99.86	99.88
CD156c (ADAM10)	99.34	98.65
CD158a/h	3.61	1.81
CD158b (KIR2DL2/L3, NKAT2)	4.1	2.73
CD158d	4.62	3.07
CD158e1 (KIR3DL1, NKB1)	0.7	0.7
CD158F	3.05	1.71
CD161	1.58	1.36
CD162	1.8	1.62
CD163	1.73	1.58
CD164	72.23	89.2
CD165	99.3	97.33
CD166	99.98	98.95
CD167a (DDR1)	3.49	2.58
CD169	0.6	0.46
CD170 (Siglec-5)	6.41	6.36
CD172a (SIRPa)	92.81	95.05
CD172b (SIRPg)	1.56	1.15
CD172g (SIRPg)	2.27	1.38
CD178 (Fas-L)	2.07	1.97
CD179a	4.52	3.09
CD179b	1.68	1.1
CD180 (RP105)	2.11	1.17
CD181 (CXCR1)	3.85	3.07
CD182 (CXCR2)	1.67	0.95
CD183	5.1	3.37
CD184 (CXCR4)	7.1	5.72
CD193 (CCR3)	3.04	1.89

CD195 (CCR5)	5.37	3.17
CD196	4.18	2.39
CD197 (CCR7)	4.64	2.83
CD200 (OX2)	99.91	98
CD200 R	3.07	1.91
CD201 (EPCR)	98.17	97.76
CD202b (Tie2/Tek)	3.45	2.97
CD203c (E-NPP3)	2.77	1.98
CD205 (DEC-205)	3.28	2.29
CD206 (MMR)	2.6	1.41
CD207 (Langerin)	1.68	1.15
CD209 (DC-SIGN)	2.3	1.1
CD210 (IL-10 R)	3.98	2.86
CD213a2	4.65	1.38
CD215 (IL-15 R alpha)	3.6	1.93
CD218a (IL-18R alpha)	4.81	2.35
CD220	32.22	29.95
CD221 (IGF-1R)	6.06	6.25
CD226 (DNAM-1)	3.47	1.61
CD229 (Ly-9)	0.69	0.57
CD231 (TALLA)	6.88	7.44
CD235ab	0.24	0.17
CD243	68.87	48.28
CD244 (2B4)	15.24	5.51
CD245 (p220/240)	12.06	11.4
CD252 (OX4OL)	4.73	3.67
CD253 (TRAIL)	2.94	1.55
CD254	16.21	12.96
CD255 (TWEAK)	18.07	11.67
CD257 (BAFF,BLYS)	56.98	55.05
CD258 (LIGHT)	15.21	15.97
CD261 (DR4, TRAIL-R1)	2.75	2.18
CD262 (DR5, TRAIL-R2)	89.8	89.17
CD263 (DcR1, TRAIL-R3)	9.14	7.25
CD266 (Fn14, Receptor TWEAK)	99.25	98.76
CD267 (TACI)	2.94	1.73
CD268 (BAFF-R, BAFFR)	1.56	1.18
CD270 (HVEM)	3.21	2.63
CD271	1.99	1.37
CD273 (B7-DC,PD-L2)	88.65	90.05
CD274 (B7-H1,PD-L1)	95.91	98.3

CD275 (B7-H2,B7-RP1, ICOSL)	51.05	48.92
CD276	99.73	99.56
CD277	30.26	31.36
CD278 (ICOS)	0.96	0.64
CD279 (PD-1)	18.1	6.95
CD282 (TLR2)	75.58	62.64
CD284 (TLR4)	1.61	1.25
CD286 (TLR6)	1.93	1.28
CD290	2.49	1.32
CD294	3.53	2.53
CD298	99.87	99.89
CD300e (IREM-2)	46.72	41.29
CD300F	2.33	1.43
CD301	4.76	2.59
CD303	0.96	0.62
CD304	16.06	11.21
CD307	1	1.19
CD307d (FcRL4)	3.51	2.55
CD314 (NKG2D)	7.4	5.3
CD317	7.64	11.92
CD318 (CDCP1)	51.89	81.34
CD319 (CRACC)	4.84	4.6
CD324 (E-Cadherin)	3.18	1.87
CD325	9.16	19.22
CD326 (Ep-CAM)	1.19	1.75
CD328 (Siglec-7)	1.1	0.68
CD334 (FGFR4)	1.36	1.73
CD335 (NKp46)	1.81	1.13
CD336 (NKp44)	1.12	0.98
CD337 (NKp30)	1.88	1.26
CD338 (ABCG2)	17.29	5.52
CD340 (erbB2_HER-2)	68.24	55.37
CD344 (Frizzled-4)	13.37	10
CD351	2.56	2.44
CD352 (NTB-A)	3.05	2.01
CD354 (TREM-1)	2	1.74
CD355 (CRTAM)	2.09	1.63
CD357 (GITR)	4.67	2.63
CD360 (IL-21R)	5.63	4.17
Microglobulin beta 2	99.98	99.62
BTLA	5.47	3.89

C3AR	33.01	18.95
C5L2	7.23	4.24
CCR10	5.15	3.48
CLEC12A	1.11	0.69
CLEC9A	3.46	2.35
CX3CR1	8.88	7.3
CXCR7	15	17.91
delta-Opioid receptor	2.17	1.74
DLL1	3.77	3.71
DLL4	0.39	0.33
DR3 (TRAMP)	3.47	1.73
EGFR	97.47	97.78
erbB3/HER-3	1.24	0.83
FceRl alpha	3.26	1.38
FcRL6	5.69	4.03
Galectin-9	2.22	1.92
GARP (LRRC32)	7.01	21.48
HLA-A,B,C	99.49	98.85
HLA-A2	40.58	98.35
HLA-DQ	0.52	0.34
HLA-DR	0.95	1.05
HLA-E	3.66	2.1
HLA-G	2.31	1.26
IFN-g R b chain	3.75	1.89
Ig k light chain	0.3	0.37
Ig omega light chain	0.37	0.38
IgD	2.11	2.17
IgM	0.7	0.54
IL-28RA	2.27	1.42
Integrin alpha 9 beta1	3.25	1.77
Integrin beta5	40.62	42.83
Integrin beta7	3.1	2.03
Jagged 2	4.91	3.49
LAP	1.43	1.21
Lymphotoxin beta receptor (LT-bR)	58.01	73.62
Mac-2 (Galectin-3)	4.63	2.73
MAIR-II	6.54	2.86
MICA/MICB PE	42.39	17.59
MSC(W3D5)	36.98	48.26
MSC(W5C5)	43.32	47.85
MSC(W7C6)	83.25	84.13

MSC and NPC(W4A5)	23.31	14.04
MSCA-1(MSC,W8B2)	27.37	24.37
NKp80	2.01	1.53
Notch1	2.02	1.84
Notch2	5.61	4.48
Notch3	2.63	2.56
Notch4	4.44	3.63
NPC(57D2)	4.83	2.53
Podoplanin	17.38	11.96
Pre-BCR	9.62	16.57
PSMA	4.41	7.35
Siglec-10	3.63	2.67
Siglec-8	3.71	1.75
Siglec-9	1.56	1.31
SSEA-1	0.87	0.46
SSEA-3	9.24	6.97
SSEA-4	49.04	29.26
SSEA-5	6.1	3.54
TCRg/d	8.72	4.67
TCR Vbeta13.2	2.51	1.74
TCR Vbeta23	6.27	7.81
TCR Vbeta8	3.34	2.4
TCR Vbeta9	1.36	0.86
TCR Vdelta2	4.31	2.11
TCR Vg9	4.52	2.95
TCR V alpha 24-J alpha18	4.3	2.44
TCR V alpha 7.2	3.41	2.06
TCR alpha/beta	5.51	3.38
Tim-1	3.81	2.48
Tim-3	2.09	1.8
Tim-4	3.43	2.57
TLT-2	4.08	2.57
TRA-1-60R	0.83	0.45
TRA-1-81	1	0.57
TSLPR (TSLP-R)	3.02	2.11

EXAMPLE 6: *Comparative study with respect to the cardiac cell isolation methods of the prior art*

A comparison was made between the number of cells, the purity and the cell viability, of the cell population obtained with the method according to the

invention with respect to the prior art [4].

In the background the selection is performed using the Facs Cell Sorter (e.g., using the tool FacsAria BD) [4]. But, in this case it is only possible to select the population of cells that expresses at high levels the antigen used for selection (bright population), while it is not possible to obtain also the positive cells that express the antigen of interest but at low levels (positive Dim population).

With the method according to the present invention, it is possible to select all the cells that express the antigen of interest, regardless of the level of expression thereof.

Figure 12 illustrates a diagram of the isolation method according to the invention that also allows the population to be selected that expresses the antigen of interest (c-kit) characterized by an intermediate or low mean fluorescence intensity (panel a). Panel b) shows a table comparing the number of cells, the purity and the cell viability, of the cell population obtained with the method according to the invention with respect to the prior art [4].

EXAMPLE 7*: Phenotypic and functional characterization of human cardiac progenitor cell CD90$^-$ and CD117$^+$ / CD90$^-$ subpopulations obtained by the method of the invention.*

MATERIALS AND METHODS

Patients and tissue samples

Right human auricle samples were obtained from patients undergoing elective cardiac surgery procedures. The informed consent previously approved by the local ethic committee was obtained for each patient in accordance with the Declaration of Helsinki.

Flow cytometry (FACS)

Immunophenotypic analysis of mesenchymal, haematopoietic and inflammatory markers was performed using multicolor flow cytometry on hCPC-CD90$^-$ cells. After detachment using a non-enzymatic solution, cells were resuspended in PBS containing 0.1% of BSA (Gibco, USA) and 2 mM EDTA (Gibco, USA) and incubated in the dark for 15 minutes with appropriate combinations of the following monoclonal antibodies or corresponding isotype: CD29-PE, CD44-PE,

CD73-PE, CD105-APC, CD14 FITC, CD34-FITC, CD45-PE, HLA-DR-FITC, CD146-FITC (BD Pharmingen, Italy), CD200-FITC, KDR-PE (R & D Systems, USA) and CD144-Alexa700 (16B1 clone; eBioscience). The samples were then washed with 1 mL of wash buffer and centrifuged for 10 minutes at 400 x g at 4° C to remove unbound antibodies. The cells were resuspended in 250 μL of washing buffer and analyzed at the flow cytometer.

Endothelial functional assay and differentiation

To evaluate the ability of hCPC-ns cells and the different subpopulations derived from them to form vascular structures *in vitro*, the cells were seeded on the basal membrane of Cultrex (Trevigen, USA). Cultrex was allowed to polymerize onto 48-well plates at 37°C, 5% CO_2 for 30 min. Cells were detached using a non-enzymatic solution, counted, diluted to $8x10^4$ cells/ml in complete endothelial growth medium-2 (EGM-2, Lonza, Italy) and seeded to each well containing the cultrex matrix. The plates were incubated at 37 ° C, 5% CO2 for 4 hours after which the number of capillary structures and the number of their branching points were counted. As a positive control in these experiments, HUVEC cells (Lonza, Italy) were used under the same culture conditions. Endothelial commitment was analyzed by culturing hCPC-ns and the different subpopulations derived from them (hCPC-CD117 +, hCPC-CD90-) for 3 weeks in EGM-2 after which the immunophenotype was determined by flow cytometry.

Cytokine secretion in the culture medium

To determine the expression of cytokines in the culture medium, the conditioned medium of hCPC-ns cells and the different subpopulations derived from them ($hCPC\text{-}CD117^+$, $hCPC\text{-}CD90^+$, $hCPC\text{-}CD90^-$) was collected to measure the amount of soluble factors released in the culture medium within 48 hours. Subsequently, a microsphere-based multiplex immunoassay (Bio-Plex assay, Bio-Rad Laboratories) was used to compare the cytokines, chemokines and growth factors released in the culture medium. The culture medium was centrifuged at 4000 g for 10 minutes. The supernatant was collected and frozen at 80 ° C until use. The samples were evaluated in duplicate due to the presence of the following angiogenic factors: the stromal cell-derived factor (SDF-1), the GRO (growth-

regulated oncogene)-alpha (GRO-α), the stem cell factor (SCF) , the leukemia inhibitory factor (LIF), interleukin-6 (IL-6), IL-8, IL-10, the monocyte chemoattractant protein 1 (MCP-1), the macrophages inflammatory protein 1 beta (MIP-1b), the Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), using the Luminex technology (Bio- Plex, Bio-Rad), according to the instructions for use.

Analysis of cardiac levels of Troponin-I (cTnI)

To determine the concentration of cTnI in the cardiomyocytes derived from iPs (CM-d-hiPSCs), a co-culture in transwell® (pore 0.4μm) was set up between CM-d-hiPSCs and hCPC-ns cells (and the different subpopulations derived from them, hCPC-CD117$^+$, hCPC-CD90$^+$, hCPC-CD90$^-$). After 7 days from the beginning of the co-culture, the conditioned medium was collected and used for the cardiac troponin ELISA assay. The conditioned medium was centrifuged for 10 minutes at 4000 g and the supernatant used for the determination of cTnI concentration, using the chemiluminescent ELISA kit (Calbiotech) according to the instructions contained in the kit.

Analysis of TNF-α

To determine the TNF-α concentration in cardiomyocytes derived from iPs (CM-d-hiPSCs), a co-culture in transwell® (pore 0.4μm) was set up between CM-d-hiPSCs and hCPC-ns cells (and the different subpopulations derived from them, hCPC-CD117$^+$, hCPC-CD90$^+$, hCPC-CD90$^-$). After 3 and 7 days from the beginning of the co-culture, the conditioned medium was collected and after centrifugation (10 'to 4000g) used for the ELISA (Invitrogen) assay, according to the instructions contained in the kit. To normalize the TNF content on the protein concentration of each sample on the same conditioned medium used for the ELISA assay, the protein dosage was also carried out using BCA (Pierce), measuring the absorbance at 540 nm.

Sircol assay

The content of total soluble collagen in cell lysates and supernatant of hCPC-ns and the different subpopulations derived from them, hCPC-CD117$^+$, hCPC-

$CD90^+$, hCPC-$CD90^-$, treated with TGF-β1 for 5 days, was measured using sircol assay (Biocolor) as described in the manufacturer's protocol. The amount of collagen was calculated according to a standard curve.

Statistical analysis

Quantitative results are expressed as mean ± standard deviation (SD) or standard error (SE). The variables were analyzed by the Student's t test. Statistical significance was evaluated with GraphPad Prism 5 and a value of $P \leq 0.05$ was considered as statistically significant.

RESULTS

Recently, it has emerged the idea that stem cells and/or progenitor cells used in cell therapy have a positive effect on the damaged myocardium through the production of soluble factors exerting a cardioprotective and anti-apoptotic action, increasing angiogenesis and modulating the inflammatory process. It is therefore crucial to find a cell population that is able to efficiently modulate these processes.

Immunophenotypic characterization of hCPC-$CD90^-$ cells

In agreement with that previously reported [4], the subpopulation of hCPC-$CD90^-$ cells equally maintains the phenotypic characteristics of mesenchymal cells, expressing typical mesenchymal markers (for example CD29, CD44, CD73 and CD200), and not expressing hematopoietic (eg CD14 and CD34) and immune system markers (HLA-DR). Table 3 shows the characterization of hCPC-$CD90^-$ at flow cytometry and indicates the expression of mesenchymal (CD29, CD44, CD73, CD105 and CD200), immune system (HLA-DR) and hematopoietic markers (CD34, CD45 and CD14). Data are expressed as mean ± SE (n = 9).

Table 3

MARKER	CPC $CD90^-$ Cells
CD29	97.20 ± 3.19
CD44	97.46 ± 0.37
CD73	95.75 ± 3.68
CD105	96.92 ± 0.59
CD200	90.18 ±15.15

HLA-DR	0 ± 0
CD34	1.76 ± 0.52
CD45	0.64 ± 0.28
CD14	90.18 ± 15.15

Endothelial differentiation of hCPC-CD90$^-$ cells

The differentiation of hCPC-CD90$^-$ cells was evaluated by functional assays and flow cytometry. After expansion, cells were tested for the ability to form tubular structures on the cultrex synthetic matrix, showing a significant increase in the number of new tubular structures ramifications after 4h with respect to the other analyzed populations ((hCPC-CD117$^+$, hCPC-CD90$^+$, hCPC-CD90$^-$) (Figure 18). Furthermore, hCPC-CD90$^-$ cells were grown in pro-angiogenic medium EGM-2 for 3 weeks to establish their ability to differentiate into mature endothelial cells. Results showed that hCPC-CD90$^-$ are more prone to differentiate into endothelial cells as indicated by the increased expression of endothelial markers CD144/Ve-cadherin and KDR/VEGFR2 compared to other considered populations of hCPC (hCPC-ns and hCPC-CD117$^+$), reaching statistical significance when compared to hCPC-CD117$^+$ cells (Figure 19).

Analysis of cytokine, chemokine and growth factor secretion by hCPC-CD90$^-$ cells

The pro-angiogenic potential of different hCPC sub-populations was tested through the use of multiplex analysis by comparing the cytokine content in the supernatant of hCPC-ns, hCPC-CD117$^+$, hCPC-CD90$^+$ and hCPC-CD90$^-$. As shown in Table 4, a number of angiogenic cytokines were found in the supernatant of hCPC and sub-populations derived from them: SDF-1, Gro-α, SCF, LIF, IL-6, IL-8, IL-10, MCP- 1, MIP-1b, RANTES, HGF and VEGF.

Table 4

Cytokines	hCPC-ns	hCPC-CD90$^+$	hCPC-CD90$^-$
Sdf-1α	20.20±4.03	25.87±9.45	54.38±30.54
Gro-α	142±35.71	380.3±108.9	425.4±82.91*

SCF	0.03±0.01	0.024±0.002	0.078±0.05
LIF	0.617±0.44	0.65±0.12	0.613±0.23
IL-6	555.8±158.7	1289±471.5	1679±276.7*
IL-8	97.38±23.21	261.8±76.93	383.4±96.02
IL-10	0.007±0.005	0.011±0.009	0.129±0.013
MCP-1	188.1±2.35	432.5±124.8	685.2±149.2**
MIP-1b	0.085±0.046	0.267±0.109	0.326±0.038*
RANTES	0.083±0.083	0.06±0.06	0.094±0.11
VEGF	30.71±14.07	34.89±1.34	41.35±19.22
HGF	566±324.5	676.5±29.7	1257±886.2

The Table indicates the expression of each cytokine in pg/ml/10^5 released by the cells in 48 hours and the results of the statistical comparison of factors produced by the 3 cell types with Student t-test. *=comparison respect to hCPC-ns; $p < 0.05$ with t-test. Data are expressed as mean ± SE (n = 3).

In particular, we found that the levels of pro-angiogenic (Gro-α and IL-8) and pro-inflammatory cytokines (IL-6, MCP-1 and MIP-1b) were significantly enriched in the supernatant of hCPC-CD90$^-$ compared to the unselected population (hCPC-ns) of the same patients, suggesting that the selected population shows a greater ability to produce factors compared to the unselected population of the same sample. Moreover, we analyze the secretion factor enrichment in hCPC-CD117$^+$, hCPC-CD90$^+$ and hCPC-CD90$^-$ subpopulations, vs the unselected counterpart from the same patients (Figure 20). The result shows an overexpression of almost all factors considered in the hCPC-CD90$^-$ with respect to the hCPC-CD117$^+$ subpopulation reaching the statistical significant for IL-6 and IL-8.

<u>hCPC-CD90$^-$ mediates the reversion of the main pathophysiological characteristics of Duchenne and Becker's muscular dystrophy.</u>

iPS-derived cardiomyocytes of dystrophic patients (DMD) (Duchenne and Becker dystrophy) exhibit a series of phenotypic deficits, typical of muscular dystrophy, including an increase in cardiomyocyte death and pro-inflammatory cytokine release such as tumor necrosis factor (TNF) –α, as described for other myocardial

diseases [6]. The discovery of a cell population that antagonize multiple DMD pathophysiological pathways is crucial in the perspective of a possible exploitation in cell therapy. For this reason, several populations of hCPC were cultured in the presence of iPS-derived cardiomyocytes of dystrophic patients. Supernatant was collected after 3 and 7 days from the beginning of the co-culture and used to evaluate indices of cardiac damage occurring in the dystrophic disorder: the release of cardiac troponin I and of TNF-α in culture medium.

Cardioprotective effect of hCPC-CD90$^-$ subpopulation

To evaluate the cardioprotective effect of different hCPC populations, they were co-cultured in the presence of CM-d-hiPSCs from Duchenne and Becker patients. Results showed that the subpopulation of hCPC-CD90$^-$ is the only one, among those analyzed, able to significantly decrease the death of CM-d-hiPSCs of Duchenne and Becker patients (measured by the release of cTnI in the culture) after 7 days of culture (Figure 21).

Anti-inflammatory effect of hCPC-CD90- and hCPC CD117$^+$/CD90$^-$ subpopulations

To evaluate the anti-inflammatory effect of different hCPC populations, cells were co-cultured in the presence of CM-d-hiPSCs of Duchenne and Becker patients. Results showed that the hCPC-CD90$^-$ subpopulation is the only, one among those analyzed, able to significantly mitigate the damage occurring in CM-d-hiPSCs of Duchenne patients after 3 days of culture, as demonstrated by the reduced release of TNF-α in culture medium. As for CM-d-hiPSCs of Becker patients, displaying a less damaged phenotype, they positively respond to different considered subpopulations (hCPC-CD117$^+$, hCPC-CD90$^-$ and hCPC-CD117$^+$/CD90$^-$), showing a significant decrease in TNF-α release in the culture medium. In these conditions, the cell population, that appear to induce the more significant effect, is the hCPC-CD117$^+$/CD90$^-$ subpopulation followed by hCPC CD90$^-$ subpopulation (Figure 22).

In vitro effect of hCPC treatment with TGF-β1

In the frame of cell therapy using progenitor cell of mesenchymal origin, it is necessary to look at the capability of these cells to differentiate into

myofibroblasts which produce collagen, because this unwanted phenomenon could compromise the recovery of damaged myocardium.

Indeed, although collagen deposition is an essential and, normally, a reversible part of wound healing, the physiological tissue repair can evolve into a progressively irreversible fibrotic response when the accumulation of fibrotic connective tissue occurs (such as collagen and fibronectin) leading to permanent scarring, heart failure and ultimately death, as in heart failure.

In this context, it is crucial to have cells that, even in a pro-fibrotic context (with the increase of TGF-β1expression), do not participate in the deposition of collagen. For this reason, we analyzed the effect of TGF-β1 treatment (5 days) to induce in different populations of hCPC the production of soluble collagen in the culture medium.

Results of the collagen quantification in these cells and in the culture medium by Sircol assay showed that hCPC-CD90^{-} subpopulation has the lowest ability to produce collagen compared to the other considered populations (hCPC-ns, hCPC-CD117^{+} and hCPC- CD90^{+}) (Figure 23).

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CLAIMS

1. Method for the isolation of human cardiac progenitor cells subpopulations, comprising the following steps:

a) mincing a heart tissue sample containing a population of cardiac cells;

b) 4-times progressive digestion of the cardiac tissue with an enzymatic mixture until a cell suspension is achieved and filtration is achieved through filters of size comprised between 30 μM and 100 μM;

c) further digestion of residual cardiac tissue obtained in step b) for 16 hours with an enzymatic mixture until a cell suspension is achieved and filtration through filters of size comprised between 30 μM and 100 μM;

d) culturing the cell suspension obtained in step b) and c) in a culture medium suitable to propagate the cardiac cells;

e) preliminary expansion of the cardiac cells obtained in step d) in a culture medium suitable to expand the cardiac cells through removal of the cardiac cells from the culture medium in step d) using an enzymatic or non enzymatic solution;

f) secondary expansion of the cardiac cells obtained in step e) in a culture medium suitable to further expand the cardiac cells through removal of the cardiac cells from the culture medium in step e) using an enzymatic or non enzymatic solution;

g) isolating one or more subpopulations of cardiac cells by positive and/or negative selection by the use of monoclonal antibodies directed against one or more surface antigens expressed in the starting population of cardiac progenitor cells.

2. Method for the isolation of human cardiac progenitor cell subpopulations according to claim 1, wherein said heart tissue sample of step a) is selected from the group consisting of right auricle, left auricle, septum, apex or ventricular biopsy.

3. Method for the isolation of human cardiac progenitor cell subpopulations according to anyone of the claims 1-2, wherein the mincing of step a) occurs in

manual or automatic mode.

4. Method for the isolation of human cardiac progenitor cell subpopulations according to anyone of the claims 1-3, wherein said enzymatic mixture of step b) and c) comprises a basal medium and an enzymatic solution.

5. Method for the isolation of human cardiac progenitor cells subpopulations according to claim 4, wherein said enzymatic solution comprises a mixture of collagenases and/or proteases.

6. Method for the isolation of human cardiac progenitor cells subpopulations according to claim 5, wherein said collagenase and/or protease mixture comprises one or more enzymes selected from the group consisting of collagenase I, collagenase II, collagenase IV, trypsin, EDTA, Accutase™, collagenase A, Dispase™ and Liberase™.

7. Method for the isolation of human cardiac progenitor cells subpopulations according to anyone of the claims 4-6, wherein said basal medium is Ham's/F12.

8. Method for the isolation of human cardiac progenitor cells subpopulations according to anyone of the claims 5 or 6, wherein said collagenase mixture is present in a concentration comprised between 0.1 and 3 mg/ml.

9. Method for the isolation of human cardiac progenitor cells subpopulations according to anyone of the claims 1-8, wherein said medium suitable for multiplying and expanding the cardiac cells of steps d), e) and f) is Ham's/F12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutathione and 5 x 10^{-3} U/ml human erythropoietin, 10 ng/ml of basic fibroblast growth factor and antibiotics.

10. Method for the isolation of human cardiac progenitor cells subpopulations according to anyone of the claims 1-9, wherein the enzymatic or non enzymatic solution of step e) and f) for cell expansion is selected from the group consisting of Tryple™ Select, a mixture of trypsin and/or EDTA, Cell Stripper™, Accutase™, or Dispase™.

11. Method for the isolation of human cardiac progenitor cells subpopulations

according to anyone of the claims 1-10, wherein said monoclonal antibodies of step g) are marked with biotin or a fluorescent molecule, preferably selected between FITC or APC.

12. Method for the isolation of human cardiac progenitor cells subpopulations according to anyone of the claims 1-11, wherein said antibodies of step g) are monoclonal antibodies against one or more antigens selected from the group consisting of:

CD1a	CD1b	CD1c	CD1d	CD2
CD3	CD4	CD5	CD6	CD7
CD8a	CD9	CD10	CD11a	CD11b
CD11b activated	CD11c	CD13	CD14	CD15 (SSEA-1)
CD16	CD18	CD19	CD20	CD21
CD22	CD23	CD24	CD25	CD26
CD27	CD28	CD29	CD30	CD31
CD32	CD33	CD34	CD35	CD36
CD38	CD39	CD40	CD41	CD42b
CD43	CD44	CD45	CD45RA	CD45RB
CD45RO	CD46	CD47	CD48	CD49a
CD49c	CD49d	CD49e	CD49f	CD50 (ICAM-3)
CD51	CD51/61	CD52	CD53	CD54
CD55	CD56 (NCAM)	CD57	CD58	CD59
CD61	CD62E	CD62L	CD62P (P-Selectin)	CD63
CD64	CD66a/c/e	CD66b	CD69	CD70
CD71	CD73	CD74	CD79b	CD80
CD81	CD82	CD83	CD84	CD85a (ILT5)
CD85d (ILT4)	CD85g (ILT7)	CD85h (ILT1)	CD85j (ILT2)	CD85k (ILT3)
CD86	CD87	CD88	CD89	CD90 (Thy1)
CD93	CD94	CD95	CD96	CD97
CD99	CD100	CD101 (BB27)	CD102	CD103
CD104	CD105	CD106	CD107a (LAMP-1)	CD108
CD109	CD111	CD112 (Nectin-2)	CD114	CD115
CD116	CD117 (c-kit)	CD119 (IFN-g R alpha chain)	CD122	CD123
CD124	CD126 (IL-6 R alpha)	CD127 (IL7 R alpha)	CD129 (IL-9 R)	CD131
CD132	CD134	CD135	CD137 (4-1 BB)	CD137L (4-1 BB Ligand)
CD138	CD140a	CD140b	CD141	CD143
CD143	CD146	CD148	CD150 (SLAM)	CD152
CD154	CD155 (PVR)	CD156c (ADAM10)	CD158a/h	CD158b (KIR2DL2/L3,NKAT2)
CD158d	CD158e1 (KIR3DL1,NKB1)	CD158F	CD161	CD162
CD163	CD164	CD165	CD166	CD167a (DDR1)
CD169	CD170 (Siglec-5)	CD172a (SIRPa)	CD172b (SIRPg)	CD172g (SIRPg)
CD178 (Fas-L)	CD179a	CD179b	CD180 (RP105)	CD181 (CXCR1)
CD182 (CXCR2)	CD183	CD184 (CXCR4)	CD193 (CCR3)	CD195 (CCR5)
CD196	CD197 (CCR7)	CD200 (OX2)	CD200 R	CD201 (EPCR)
CD202b (Tie2/Tek)	CD203c (E-NPP3)	CD205 (DEC-205)	CD206 (MMR)	CD207 (Langerin)
CD209 (DC-SIGN)	CD210 (IL-10 R)	CD213a2	CD215 (IL-15 Ralpha)	CD218a (IL-18R alpha)
CD220	CD221 (IGF-1R)	CD226 (DNAM-1)	CD229 (Ly-9)	CD231 (TALLA)
CD235ab	CD243	CD244 (2B4)	CD245 (p220/240)	CD252 (OX4OL)
CD253 (Trail)	CD254	CD255 (TWEAK)	CD257 (BAFF,BLYS)	CD258 (LIGHT)
CD261 (DR4,TRAIL-R1)	CD262 (DR5,TRAIL-R2)	CD263 (DcR1,TRAIL-R3)	CD266 (Fn14,TWEAK R	CD267 (TACI)
CD268 (BAFF-R,BAFFR)	CD270 (HVEM)	CD271	CD273 (B7-DC,PD-L2)	CD274 (B7-H1,PD-L1)
CD275 (B7-H2,B7-RP1,ICOSL)	CD276	CD277	CD278 (ICOS)	CD279 (PD-1)
CD282 (TLR2)	CD284 (TLR4)	CD286 (TLR6)	CD290	CD294
CD298	CD300e (IREM-2)	CD300F	CD301	CD303
CD304	CD307	CD307d (FcRL4)	CD314 (NKG2D)	CD317
CD318 (CDCP1)	CD319 (CRACC)	CD324 (E-Cadherin)	CD325	CD326 (Ep-CAM)
CD328 (Siglec-7)	CD334 (FGFR4)	CD335 (NKp46)	CD336 (NKp44)	CD337 (NKp30)
CD338 (ABCG2)	CD340 (erbB2_HER-2)	CD344 (Frizzled-4)	CD351	CD352 (NTB-A)
CD354 (TREM-1)	CD355 (CRTAM)	CD357 (GITR)	CD360 (IL-21R)	beta2-microglobulin
BTLA	C3AR	C5L2	CCR10	CLEC12A
CLEC9A	CX3CR1	CXCR7	delta-Opioid Recepto	DLL1
DLL4	DR3 (TRAMP)	EGFR	erbB3/HER-3	FceRIalpha
FcRL6	Galectin-9	GARP (LRRC32)	HLA-A,B,C	HLA-A2
HLA-DQ	HLA-DR	HLA-E	HLA-G	IFN-g R b chain
Ig light chain k	Ig light chain omega	IgD	IgM	IL-28RA
Integrin alpha9beta1	Integrin beta5	Integrin beta7	Jagged 2	LAP
Lymphotoxin beta receptor (L	Mac-2 (Galectin-3)	MAIR-II	MICA/MICB PE	MSC(W3D5)
MSC(W5C5)	MSC(W7C6)	MSC and NPC(W4A5)	MSCA-1(MSC,W8B2)	NKp80
Notch1	Notch2	Notch3	Notch4	NPC(57D2)
Podoplanin	Pre-BCR	PSMA	Siglec-10	Siglec-8
Siglec-9	SSEA-1	SSEA-3	SSEA-4	SSEA-5
TCRg/d	TCR Vbeta13.2	TCR Vbeta23	TCR Vbeta8	TCR Vbeta9
TCR Vdelta2	TCR Vg9	TCR Valpha24-Jalpha18	TCR Valpha7.2	TCR alpha/beta
Tim-1	Tim-3	Tim-4	TLT-2	TRA-1-60R
TRA-1-81	TSLPR (TSLP-R)			

13. Method for the isolation of human cardiac progenitor cells subpopulations according to anyone of the preceding claims wherein the selection of step g) is carried out by immunomagnetic separation.

14. Method for the isolation of human cardiac progenitor cells subpopulations according to anyone of the claims 11-13, wherein said monoclonal antibodies of step g) are anti-CD117 and/or anti-CD90.

15. Subpopulation of human cardiac progenitor cells from human auricle obtained by the method of isolation according to claim 14 exclusively by negative selection using anti-CD90 monoclonal antibodies, characterized by a surface marker profile $CD90^-$; by expressing mesenchymal markers CD29, CD44, CD73, CD105 and CD200; by not expressing hematopoietic markers CD34, CD45, CD14 and the immune system marker HLA-DR.

16. Subpopulation of human cardiac progenitor cells according to claim 15, for use in the treatment of cardiovascular diseases, alone or in combination with another subpopulation of human cardiac progenitor cells.

17. Subpopulation of human cardiac progenitor cells according to claim 16, for use in cardiac cell and/or tissue transplant, alone or in combination with another subpopulation of human cardiac progenitor cells.