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(54) Title: MUSCLE-DERIVED CELL POPULATIONS WITH CARDIOGENIC DIFFERENTIATION CAPACITIES

(57) Abstract: The present application relates to cell populations derived from skeletal muscle tissues, and which have cardiogenic differentiation capacities. The invention also relates to a method for committing a cell population derived from skeletal muscle tissues to a cardiogenic cell population and to compositions for implementing said method.



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MUSCLE-DERIVED CELL POPULATIONS WITH CARDIOGENIC DIFFERENTIATION CAPACITIES

FIELD OF THE INVENTION

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The present application relates to cell populations derived from skeletal muscle tissues, and which have cardiogenic differentiation capacities. The invention also relates to a method for committing a cell population derived from skeletal muscle tissues to a cardiogenic cell population and to compositions for implementing said method.

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BACKGROUND OF THE INVENTION

Heart failure represents one of the main causes of morbidity and mortality in the 21st century. In France, it affects approximately 500 000 patients, counting more than 120 000 new cases per year. The rate of death within the five years following the diagnosis is more than 50%. The main causes of heart failure are myocardial ischemia (infarction, coronary disease) and dysfunctions linked to gene mutations (dilated and/or hypertrophic cardiomyopathies). In half cases, heart failure is then triggered by a coronary ischemic accident leading to subsequent hypoxia and inflammatory processes responsible for the rapid and irreversible loss of contractile adult cardiomyocytes. An akinetic cicatricial fibrous tissue (scar) takes place of contractile myocardium and leads to a progressive deleterious remodeling. Pharmacological treatments (Angiotensin-converting enzyme inhibitors, diuretics, beta-blockers) have dramatically improved the prognosis, however, they remain symptomatic ones, and patients might become refractory. Heart transplantation is limited by the shortage of donors. Long-term implantable left ventricle assist devices are under development. Therefore, cell transplantation is very actively investigated as a strategy to improve cardiac structure and function. This biological approach is expected to (1) replace the scar tissue by a living tissue with better physical properties, (2) block or revert the remodeling process, (3) restore myocardial contractility, (4) induce local angiogenesis and improve perfusion and (5) recruit hibernating cardiomyocytes or cardiac stem cells. The autologous approach is most often employed to avoid immunological rejection. Many cell types have been proposed and/or tested. Their usefulness is frequently limited by (1) the soundness of identification and characterization, (2) the easiness of procurement, (3) the capacity of being expanded ex vivo, (4) the real biological properties and (5) the way of administration.

In attempting to achieve cardiac cell-based replacement therapy, a variety of cells have been contemplated mainly in the setting of ischemic cardiomyopathy including skeletal myoblasts (Murry et al.; Pouly et al.; Menasche et al.), bone marrow mononuclear cells (Ghodsizad et al.; Ishida et al.), circulating endothelial progenitor cells (Arguero et al.; Ogawa et al.), mesenchymal cells (Bartunek et al.; Nagaya et al.), cardiac progenitors of cardiac origin (Chugh et al.). However, the routine use of these cell types in the clinic is hampered either by ethical or practical limitations or by the failure of these cells to differentiate in vivo into mature cardiomyocytes. True cardiac stem cells are rare, and often located in anatomical areas that preclude their extraction in view of autologous expansion and administration to the patient. The limitations of these adult stem cells have generated a great interest in embryonic stem cells (ES) and induced pluripotent stem cells (iPS) whose pluripotentiality allows driving their fate towards a given lineage (Kawamura et al.). However, the clinical use of ES cells is still limited by their allogenicity (raising immunological rejection; Nussbaum et al.), cell survival, the level of purity in cardiac-committed cells and the karyotype stability (both warranting the absence of tumorigenicity), and the extent of terminal differentiation (warranting electrical stability or true limitation of cardiac remodeling) (Yamada et al.; Menasche 2009; Swijnenbourg et al.), however solutions to these issues are emerging, some of which including the uses of pro-survival factors and/or supportive patches (Bel et al.; Blin et al.; Kalfa et al.). Finally, iPS are obtained upon the integration of transgenic sequences, some of which being potentially carcinogenic.

Taken together, the mitigated results of the first wave of clinical trials, and the limitations of the candidate cell types used for the cell therapy approaches, justify the researches of new cell populations that would exhibit cardiogenic capacities and the proper integration within the cardiac tissue. In addition, such cell populations could become tools to evaluate heart formation and regeneration in normal and pathological contexts (such as congenital heart diseases, Fallot tetralogia, cardiac healing after an ischemic accident...), and to attempt screening of molecules impacting heart formation, function and homeostasis (such as beta-blockers, calcium channel blockers, adrenergic drugs, ...). This raises this requirement to characterize and define new cell types with cardiac capacities. Ideally, these cells should be of easy access within the body, even in an autologous context, they should be easy to characterize, easy to prepare without the need of adding exogenous genetic material, and they should be expandable without loss of their capacities. In vitro as well as in vivo, they should exhibit the characteristics of cardiac cells.

The presence of cell populations presenting cardiogenic capacities within the skeletal muscle tissues has been suggested by different groups. The cell types are differing from those of the present invention on the basis of the process of extraction, the expression of specific markers,
5 or the nature of the experimental animal models.

Indeed most studies are based on cells of rodent origins (Mouse, Rat) and use markers that do not have real counterpart in primates (i.e. Sca-1 antigen) or that do not label the same cell types (i.e. CD34). For example, Abdel-Latif et al., Arsic et al., Shibuya et al., Zuba-Surma et al., studied mouse cells based on the expression of Sca-1. Moreover, from one study to the
10 other, the same markers would not define cells with the same properties, i.e. Sca1⁺ cells are claimed to possess a cardiogenic capacity in some studies (Shibuya et al.), while the Sca1-cells are claimed to be the most cardiogenic in other studies (Abdel-Latif 2008; Clause et al; Winitsky et al. ; Zuba-Surma et al.). Similarly, CD34 expression is required in some studies, and excluded in another from the same group (Tamaki et al., 2008; 2010).

Several groups produce their cells on the basis of a claimed low adherence (Arsic et al.; Clause et al.; Winitsky et al.). In some studies, specific treatments of cells are required to coax a cardiogenic differentiation, for example, co-cultures on mouse cells (Iijima et al. ; Tamaki et al. 2008), three-dimensional gel bioreactor (Clause et al.), cardiospheres, formation of
15 myospheres (Arsic et al.; Nomura et al. 2008; Poulet et al. ; Tamaki et al., 2010). In some cases, supplementary to co-culture, beating is required for the cardiac differentiation of some cell types (Iijima et al.). In most studies, the expression of some cardiac markers has been assessed in vitro only, and there was no experimental demonstration of integration in vivo upon intramyocardial implantation (Abdel-Latif et al., Arsic et al., Clause et al., Iijima et al., Nomura et al., Payne et al., Poulet et al.; Zuba-Surma et al.). In one study, mouse cells were
20 first grown in a 3D collagen substrate or transplanted as spheres (Tamaki et al.), which is not compatible with a clinical use.

Very few studies addressed the cardiac differentiation of Human skeletal muscle cells in vivo. One report used expanded cells and a treatment with retinoic acid (Invernici et al.), however the number of cells produced was limited. One report is based on the selection of Human
30 muscle CD34⁺ cells, which however did not directly differentiated into cardiac tissue (Proksch et al.).

In addition, several attempts to use skeletal muscle progenitors for cardiac tissue reconstruction have failed, although some functional benefits have been reported in some models or clinical trials (Menasche et al., 2008, 2009). Myoblasts and muscle-derived stem

cells generally participate to neoangiogenesis and reduce fibrosis, however the structures formed mainly comprise myotubes or small muscle fibers which do not differentiate into bona fide cardiomyocytes (Reinecke et al.; Hagege et al.; Oshima et al.) and do not participate to electromechanical junctions with resident cardiac cells (Reinecke et al.; Leobon et al.).

5 Human myoblasts and mouse muscle-derived stem cells participate to myocardial healing through paracrine mechanisms (Perez-Ilzarbe et al.).

At variance with direct skeletal muscle progenitors, stem cells from alternative compartments of the body have been tested, but the results of clinical trials have been disappointing (Menasche 2009, 2011; Jiang et al.). Hematopoietic stem cells do not differentiate into bona fide cardiomyocytes (Murry et al. 2004). Embryonic stem cells are able to integrate functionally and biologically within harmed myocardium, but their use is still limited by their procurement, the control of their differentiation status and invasiveness, some lack of biological maturation and the risk of arrhythmogenicity, their immune rejection (Menasche 2009, 2011; Zhang et al.). Because of their paucity, mesenchymal stem cells require bone marrow aspiration and long-term expansions. Nevertheless, upon treatment with cytokines the MSC may differentiate into cardiac cells *in vitro* and *in vivo* (Bartunek et al.).

A readily, easily and rapidly available human skeletal-muscle derived cell population is thus still much needed in the therapeutic field.

20 Aldehyde dehydrogenases (ALDH) constitute a large and ancient family of intracellular enzymes involved in oxidation of aliphatic and aromatic aldehydes into the corresponding acids, thereby are considered as general detoxifying enzymes eliminating toxic biogenic and xenobiotic aldehydes in Humans (Sophos et al. Yoshida et al.). Some isoenzymes allow resistance to anti-cancer drugs of the oxazaphosphorine family by their detoxification (Kastan et al; Hilton et al.). ALDH1 are especially involved in the production of retinoic acid from retinal, and therefore would play a pivotal role in retinoid-dependent differentiation pathways (Sobreira et al.).

30 These cells are observed within human bone marrow (BM) (Sophos et al.; Jones et al.; Gentry et al.; Lioznov et al.), umbilical cord blood (UCB) (Christ et al.; Storms et al.; Gentry et al.; Hess et al.; Storms et al.; Mirabelli et al., and peripheral blood (PB) (Lioznov et al.; Fallon et al.; Povsic et al.). These ALDH^{br}/SSC^{lo} are rare cells, with frequencies not exceeding 3-4 % of the mononucleated cellular fraction of the tissue source. High ALDH activity is a hallmark of cells harboring high myeloid, lymphoid, erythroid differentiation ability, and short and/or

long-term hematopoietic reconstitution capacities *in vivo* (Gentry et al.; Hess et al.; Mirabelli et al.; Liu et al.). Because of their intrinsic properties, cell populations expressing ALDH represent an expanding category of stem cells. ALDH are expressed by primitive progenitors, whose biology is still poorly described, and which have been identified in several tissues.

5 Neural, liver, pancreatic, epithelial and angiogenic progenitors have been described (Capoccia et al.; Corti et al.; Zhou et al.). The presence of cell populations expressing ALDH within cardiac tissues has been also unveiled recently by other groups (Koninckx et al.; Roehrich et al.).

10 We have previously identified populations of cells expressing one or several ALDH enzymatic activities starting from human skeletal muscle fragments, and combining flow cytometry and immunohistochemistry (Vauchez et al., EP 2 206 774). Two sub-populations have been described based on the co-expression (or its absence) of the CD34 molecule (ALDH⁺/CD34⁺ and ALDH⁺/CD34⁻) and they harbor different phenotypic and functional

15 characteristics. ALDH⁺/CD34⁺ have a mesenchymal profile, while ALDH⁺/CD34⁻ have a myogenic capacity *in vitro* and participate to muscle regeneration *in vivo* upon intramuscular administration. When placed in osteogenic medium, ALDH⁺/CD34⁻ cells express osteogenic markers. The presence in skeletal muscle of cell populations expressing ALDH has been confirmed by other groups (Jean et al.; Vella et al.) who suggested that this expression was

20 conferring a survival advantage when confronted to oxidative stress *in vitro* or *in vivo*.

The ALDH⁺/CD34⁻ form a new group of muscle progenitors and could be considered as a tool for cell therapy of degenerative diseases. Our observations suggest that the differentiation of some ALDH⁺ populations may be coaxed by the local, environmental cues, or by culture

25 conditions.

SUMMARY OF THE INVENTION

We describe herein the preparation, characterization and use of cell populations extracted

30 from skeletal muscle tissues to produce cardiomyocyte-like cells. The cell populations provided herein are able to form bona fide cardiac tissue *in vivo* after transplantation. We thus herein describe for the first time a methodology to obtain cell populations having such capacities.

An object of the invention is to provide a skeletal muscle-derived cell population having cardiogenic capacities, and compositions comprising the same. In particular, we herein describe an ALDH⁺/CD34⁻ cell population derived from skeletal muscle, this population being able to produce a cardiogenic population.

- 5 The invention also relates to a skeletal muscle-derived cell population having cardiogenic capacities for use in treating a cardiac defect, disease or pathology.

In addition, the invention relates to a cell population containing skeletal muscle-derived cells for use in the production of a cardiogenic population in a subject in need thereof. The
10 cardiogenic population is more particularly obtained by *in vitro* culturing an ALDH⁺/CD34⁻ cell population derived from skeletal muscle in a culture medium containing one or more differentiation and/or growth factor such as b-FGF, BMP-2, BMP-4 and IGF-1. In a particular embodiment, the culture medium contains b-FGF, BMP-2, BMP-4 and IGF-1.

- 15 The cell population for use according to the invention may in particular be an ALDH⁺/CD34⁻ cell population.

A further object relates to methods for isolating and enriching the cell population according to the invention. In particular, a method for the production of a cell population having
20 cardiogenic differentiation capacities is disclosed, comprising providing a skeletal muscle-derived cell population as defined herein, and culturing said population *in vitro*, thereby obtaining a cell population having cardiomyogenic differentiation abilities. Accordingly, the invention further relates to a skeletal muscle-derived cell population having cardiogenic differentiation abilities, wherein said cell population is obtainable by a method as defined in
25 the present application.

A further object of the invention corresponds to a cardiogenic cell population derived from a skeletal muscle-derived cell population. In particular, the cardiogenic cell population is obtainable according to the method herein described. In particular, the invention relates to
30 a cardiogenic cell population having the ability to form bona fide cardiac tissue *in vivo* after transplantation, said cardiogenic population being obtainable by *in vitro* culturing a skeletal muscle-derived cell population (preferably an ALDH⁺/CD34⁻ skeletal muscle-derived cell population). Particular embodiments of the method for obtaining the cardiogenic cell

population of the invention from skeletal muscle are provided throughout this application, in particular in the examples.

Another object of the invention relates to a composition useful for coaxing *in vitro* a cell population having cardiogenic capacities. In particular, the composition comprises one or more differentiation and/or growth factors such as BMP-2, BMP-4, IGF-1 and bFGF.

A further object of the invention comprises a method for isolating a skeletal muscle-derived ALDH⁺ cell population having a cardiogenic potential, comprising selecting ALDH⁺ cells by sorting skeletal muscle cells in a buffer comprising a fluorescent synthetic substrates of ALDH such as BODIPY-AAA, and a calcium channel blocker such as verapamil.

Further objects and advantages of the invention will be apparent from the detailed description and examples provided below.

LEGENDS TO THE DRAWINGS

Figure 1. Flow cytometry phenotypic characterization of enzymatically-dissociated Human muscle cells. The cells were enzymatically dissociated from Fascia Lata muscle biopsies of 7 patients, aged 47-63 year, then kept frozen. Following thawing, the cells were incubated with PE-labeled antibodies directed against the indicated surface antigens (CD9-CD309), then analysed using a FACSCalibur apparatus (Becton-Dickinson). For each marker, a cytogram was drawn representing the side scatter (SSC) as a function of PE fluorescence intensity. For each marker, the region of interest, containing the highest and discrete fluorescence intensity, was defined and allowed evaluating the proportion of cells harboring it. Of note, the number of cells expressing the marker, and the fluorescence intensity (reflecting the total number of marker molecules expressed by a given cell) varied between markers. Representative cytograms are shown. Data are presented as percentages (mean, SD) of cells positive for the indicated marker.

Figure 2. Flow cytometry phenotypic characterization of Human dissociated muscle cells expressing ALDH. The cells were enzymatically dissociated from Fascia Lata muscle biopsies of 7 patients, aged 47-63 year, then kept frozen. Following thawing, the cells were incubated with Aldefluor substrate with or without the specific inhibitor DEAB, then with PE-

labeled antibodies directed against extracellular markers, and with APC-labeled anti-CD34 antibody. The presence of Aldefluor is detected using the FL1 channel, the presence of PE-labeled markers is detected using the FL2 channel, the presence of APC-labeled CD34 is detected using the FL4 channel. Representative cytograms are shown.

5 In (A), SSC is presented as a function of FL1 intensity; left panel: in the presence of Aldefluor and DEAB, the baseline fluorescence was established and a gate was defined to eventually quantify the ALDH⁺ cells; right panel: in the presence of Aldefluor alone, a shift in fluorescence intensity (FL1 channel) defined the population of cells expressing ALDH⁺.

10 In (B), the expression of PE-labeled markers (FL2 channel) is represented as a function of ALDH⁺ cells (FL1 channel). On the left, the upper right square delineates the population of cells expressing both ALDH and the marker under scrutiny. For each marker, the value is translated on a global histogram (right panel) that represents the proportion of cells expressing the given marker within the total population of cells expressing ALDH.

15 In (C) the expression of APC-labeled CD34 marker (FL4 channel) is represented as a function of ALDH⁺ cells (FL1 channel), providing an upper right quarter containing the ALDH⁺/CD34⁺ cells, and a lower right quarter containing the ALDH⁺/CD34⁻ cells in the left panel. In each quarter, the proportion of cells associated to the expression of a PE-labeled fluorophore can be estimated. The values associated to the ALDH⁺/CD34⁻ population are translated in right panel. Data are percentages (mean, SD) of positive cells for the indicated
20 markers.

Figure 3. Flow cytometry characterization of ALDH⁺/CD34⁺ and ALDH⁺/CD34⁻ populations of Macaque and Human muscle cells. Dissociated cells from heart and quadriceps muscle biopsies of Macaques (n = 4, aged 5-9 year) and from Human fascia lata muscle
25 biopsies (n=7, aged 47-63 years) were incubated with Aldefluor substrate, then with APC-labeled anti-CD34 antibody. Representative cytograms are shown. In (A), the expression of CD34 is represented as a function of ALDH⁺ cells, providing an upper right quarter containing the ALDH⁺/CD34⁺ cells (green color), and a lower right quarter containing the ALDH⁺/CD34⁻ cells (red color), in which the proportions of cells can be estimated. In (B),
30 the assigned cell populations are projected according to their side scatter (SSC) and forward scatter (FSC). Skeletal ALDH⁺ CD34⁻ cells were the most homogenous population. Populations are indicated by arrows. Datas are percentages (mean, SD) of cells positive for the indicated marker.

Figure 4. Flow cytometry phenotypic characterization of Macaque (n = 4) muscle cells populations extracted from myocardial (ventricle, atria) and skeletal (SKM) muscle tissues. The enzymatically dissociated cells were incubated with Aldefluor substrate, then with APC-labeled anti-CD34 antibody and a second PE-labeled marker. The values associated to the combinations of these markers by the cells are translated in histograms. The proportion of a dedicated marker among the whole content of mononucleated cells is presented as the first column (empty). The proportions of cells expressing a dedicated marker within the ALDH⁺/CD34⁺ population and within the ALDH⁺/CD34⁻ population are presented in grey and in black, respectively. This representation suggests that, for example, CD9 and CD184 are especially co-expressed with ALDH⁺/CD34⁻ cells from skeletal muscles, while CD49e and CD140b are frequently associated to the ALDH⁺/CD34⁺ populations. Data are percentages (mean, SD) of positive cells for the indicated markers.

Figure 5. Enrichment of Macaque ALDH⁺ skeletal muscle cells using Ficoll. Muscle cells from 5 Macaques aged 5-20 year were enzymatically-dissociated, and labeled with or without prior centrifugation onto a Ficoll gradient. Untreated cells (A) and cells collected in the pellet (B, left panel) or at the interface (B, right panel) were incubated with Aldefluor substrate. The labelings were analysed as described previously. Cytograms were drawn representing the side scatter (SSC) as a function of fluorescence intensity (FL1 channel). Data are percentages (mean, SD) of ALDH⁺ cells.

Figure 6. Expression of Islet-1, GATA-4 and Nkx2.5 by stimulated Macaque total skeletal muscle cells. Enzymatically-dissociated Macaque muscle cells were grown in culture as a crude preparation, i.e. without sorting step in presence of one, two, three or four factors of the cocktail for 48 hours before analysis. After 48 hours, cells were harvested by trypsinization, fixed and permeabilized. The cells were incubated with rabbit antibodies directed against GATA-4, Nkx2.5 or Islet-1 then with goat anti-rabbit immunoglobulins IgG labelled with fluorophore (FITC). The cells were analyzed using a FACSCalibur apparatus and the FSC was plotted as a function of fluorescence intensity. Populations of cells expressing the cardiac markers were observed in presence of (A) bFGF alone, (B) bFGF and BMP-2, (C) bFGF and IGF-1, (D) and (E) BMP-2, IGF-1 and bFGF, (F) bFGF, BMP-2, IGF-1 and BMP-4. These are independent experiments. Data are percentages (mean, SD) of cells positive for the indicated marker.

Figure 7. Kinetic of GATA-4 and Nkx2.5 expression by stimulated Human total skeletal muscle cells. Enzymatically-dissociated Human muscle cells were grown under daily stimulation by the cocktail (BMP-4, BMP-2, IGF-1 and bFGF) (panel A), or after a single early stimulation (panel B). Cells were harvested by trypsinization, fixed and permeabilized at 48 and 72 hours. The cells were incubated with rabbit antibodies directed against GATA-4 or Nkx2.5, then with goat anti-rabbit immunoglobulins IgG labelled with fluorophore (FITC). The cells were analysed using a FACSCalibur apparatus and the FSC was plotted as a function of fluorescence intensity. Data are percentages (mean, SD) of cells positive for the indicated marker.

Figure 8. Kinetic of GATA-4 and Nkx2.5 expression by stimulated Macaque total skeletal muscle cells. As in Figure 7, enzymatically-dissociated Macaque muscle cells were grown for 48 and at 72 hours under daily stimulation by the cocktail (BMP-4, BMP-2, IGF-1 and bFGF) (panel A) or after a single early stimulation (panel B). Cells were harvested by trypsinization, fixed and permeabilized. The cells were incubated with rabbit antibodies directed against GATA-4 or Nkx2.5, then with goat anti-rabbit immunoglobulins IgG labeled with fluorophore (FITC). The cells were analysed using a FACSCalibur apparatus and the FSC was plotted as a function of fluorescence intensity. Data are percentages (mean, SD) of cells positive for the indicated marker.

Figure 9. Expression of GATA-4 and Nkx2.5 by selected and stimulated Human and Mouse skeletal muscle ALDH⁺ cells. Enzymatically-dissociated Human (upper panel) or Mouse (lower panel) muscle cells were sorted using Aldefluor substrate, then a PE-labeled anti CD34 antibody, using a FACSDIVA apparatus. ALDH⁺CD34⁺ or ALDH⁺CD34⁻ sorted cells were grown before analysis at 48 hours with a daily stimulation of the cocktail (BMP-4, BMP-2, IGF-1 and bFGF). Cells were harvested by trypsinization, fixed and permeabilized. The cells were incubated with rabbit antibodies directed against GATA-4 or Nkx2.5, then with goat anti-rabbit immunoglobulins IgG labelled with fluorophore (FITC). The cells were analysed using a FACSCalibur apparatus and the FSC was plotted as a function of fluorescence intensity. Data are percentages (mean, SD) of cells positive for the indicated marker.

Figure 10. Expression of cardiac markers in vitro by selected and stimulated Mouse muscle cells. In (A), enzymatically-dissociated Mouse muscle cells were sorted using a FACSDIVA

apparatus, on the basis of ALDH expression revealed by Aldefluor assay. These cells were grown in culture in presence of the cocktail of cytokines for seven days before analysis. Then, cells were fixed and incubated with antibodies directed against cardiac proteins, then with secondary antibodies coupled to fluorophores. The nuclei were counterstained using DAPI in the mounting medium. The cells were observed using a fluorescence microscope. The cells expressed the connexin-43 (green) and the alpha-actinin (red).

In (B), skeletal (1, 2) and cardiac (3, 4) tissue sections were incubated with antibody directed against the Cardiac Troponin I then with secondary antibodies coupled to fluorophores (1 and 3, negative controls, 2 and 4 positive stainings). In panels 5 and 6, enzymatically-dissociated and FACS-sorted Macaque muscle cells were grown in presence of the cocktail for 7 days before analysis. Then, cells were fixed and incubated with the antibodies (connexin 43, cardiac troponin I). The nuclei were counterstained using DAPI in the mounting medium. Pictures were reconstructed by juxtaposition of low magnification images. Scale bar: 10µm (A), (B5, B6), and 50µm (B1-4).

Figure 11. In vivo engraftment of selected and stimulated Macaque muscle ALDH⁺/CD34⁻ cells. Low magnification pictures, results obtained after 3 weeks. Enzymatically-dissociated Macaque ALDH⁺/CD34⁻ muscle cells were sorted using a FACSDIVA apparatus, on the basis of both ALDH positivity revealed by Aldefluor assay and lack of CD34 expression. The cells were grown in culture in presence of the cocktail for 48 hours before intramyocardial implantation into NOD-SCID immunodeficient mice. Three weeks after transplantation, the presence of Macaque-specific antigens (lamin A/C) and/or cardiac or skeletal muscle markers were assessed by immunohistofluorescence on heart sections. (A, left column). Large areas of specific Lamin A/C-positive nuclear membranes (green) were detected in association with specific cytoplasmic markers of cardiomyogenic differentiation such as Connexin-43 (blue) and alpha-actinin (red). (B) The almost complete lack of co-staining of serial sections using antibodies directed against the fast skeletal myosin isoform (red, right column) indicated that the newly implanted tissues were not skeletal muscle in nature. Scale bars: 40µm.

Figure 12. In vivo engraftment of selected and stimulated Macaque muscle ALDH⁺/CD34⁻ cells. Higher magnification pictures, results obtained after 3 weeks. As in Figure 10, enzymatically-dissociated Macaque ALDH⁺/CD34⁻ muscle cells were sorted and grown in culture in presence of the cocktail for 48 hours before intramyocardial implantation into NOD-SCID immunodeficient mice. Three weeks after transplantation, cells expressing

Macaque-specific lamins A/C and cardiomyogenic markers were identified by immunohistofluorescence on heart sections. Lamin A/C-positive nuclear membranes (green) were detected in association with Connexin-43 (blue) and alpha-actinin (red). The increased magnification allows comparing the lamin A/C-positive cells with neighboring, murine cardiomyocytes, underlining their similar morphologies and expression of Cx43 and alpha actinin. Scale bars: 100µm (Lines 1 and 2), 20µm (Lines 3 and 4).

Figure 13. In vivo engraftment of selected and stimulated Macaque muscle ALDH⁺/CD34⁻ cells. Results obtained after 4 weeks. As in Figure 11 and 12, enzymatically-dissociated Macaque ALDH⁺/CD34⁻ muscle cells were sorted, grown in culture, implanted into NOD-SCID immunodeficient mice. Four weeks after transplantation, heart sections were labeled for expression of Macaque-specific Lamin A/C (green), Connexin-43 (blue) and alpha-actinin (red). Macaque nuclei were observed inside cardiomyocytes. Scale bars: 50µm.

Figure 14. Comparison between Aldefluor buffer and Verapamil buffer. Skeletal muscle cells from 5 Macaques aged 5-20 year (panel A) and cardiac muscle cells from human donors (panel B) were enzymatically-dissociated as previously described. As presented above, SSC is presented as a function of FL1 intensity. The baseline fluorescence was established in the presence of Aldefluor and DEAB, and a gate was defined to quantify the ALDH⁺ cells; in the presence of Aldefluor alone, a shift in fluorescence intensity (FL1 channel) defined the population of cells expressing ALDH⁺. Two different buffers were used, the Aldefluor buffer commercially available from Stem Cell Technology™ (left panels), and a buffer containing Verapamil (right panels). Data are percentages (mean, SD) of ALDH⁺ cells.

Figure 15. Comparison between Aldefluor buffer and a range of Verapamil concentrations. As in Figure 14, muscle cells from 5 Macaques aged 5-20 year were enzymatically-dissociated and incubated in the classical Aldefluor buffer (upper panel), or in buffers containing increasing concentrations of Verapamil (lower panel). SSC is presented as a function of FL1 intensity. The baseline fluorescence was established in the presence of Aldefluor and DEAB. In the presence of Aldefluor alone, a shift in fluorescence intensity (FL1 channel) defined the population of cells expressing ALDH⁺. Data are percentages (mean, SD) of ALDH⁺ cells.

Figure 16. Flow cytometry characterization of Macaque ALDH⁺ muscle cells in presence of Verapamil buffer. The populations of enzymatically-dissociated muscle cells were extracted

from 5 Macaque aged 5-20 year, and were incubated with Verapamil buffer, then with PE-labeled anti CD34. In (A), left panel, the expression of CD34 marker (FL2 channel) is represented as a function of ALDH⁺ cells (FL1 channel), providing an upper right quarter containing the ALDH⁺/CD34⁺ cells, and a lower right quarter containing the ALDH⁺/CD34⁻ cells. A color can be assigned to the cells in each quarter. The right panel in (A) represents the same analysis considered as density zones instead of scatter plot. In each quarter, the proportion of cells can be estimated. In (B), the cell populations, assigned with specific colors, are projected according to their side scatter (SSC) and forward scatter (FSC). ALDH⁺/CD34⁻ cells were a small and refractive homogenous population. Data are percentages (mean, SD) of cells for the indicated marker.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides the preparation, characterization and use of cell populations extracted from skeletal muscle tissues to produce cardiomyocyte-like cells. The invention further provides the use of these cell populations for treating a cardiac defect, disease or pathology. Furthermore, the invention relates to a method for isolating and coaxing a cell population as described above and throughout this text, and to a composition useful for committing this cell population to a cardiogenic lineage.

Skeletal muscle-derived cell population according to the invention

The present application provides a skeletal muscle-derived cell population having cardiogenic capacities, i.e. able to form bona fide functional cardiac tissue after transplantation.

Source skeletal muscle sample or biopsy is obtained from any skeletal muscle of a mammal subject, in particular a human or non-human subject, preferably a human subject. In particular, the source muscle originates from a muscle sample or biopsy of the subject to be treated (autologous skeletal muscle) or from a different subject, preferably of the same animal species as the subject to be treated (heterologous muscle). Any type of muscle samples can be used in the process of the invention. Reference is made to the reference manual entitled "Atlas of Human Anatomy", 4th Edition; Frank H. Netter MD; Anne Lenahan Ed., Elsevier Saunders, Philadelphia, Pennsylvania, USA; USBN # 978-0-683-30492-3. The skeletal muscle tissue is harvested from an adult, an elderly, a young adult, a teenager, a kid. The

muscle tissue can be harvested from fetal skeletal muscle. The process can be advantageously implemented on a sample as small as a standard muscular biopsy, i.e. a sample of muscular tissue of about 0.05 – 1g or more, e.g. 0.2 - 15g which has been collected from one or several human(s) or from one or several non-human animal(s). Illustrative muscle biopsies comprise

5 biopsy from a muscle of the head and/or neck (such as from the mouth, the tongue, the soft palate, the pharynx, the larynx, the cervical vertebra), from a muscle of the torso (such as from the back, the vertebral column, the thoracic walls, the chest, the abdomen, the pelvis, the perineum), from a muscle of the upper limbs (such as from the shoulder, the arms, the forearms, the hand), from a muscle of the lower limb (such as from the iliac region, the

10 buttocks, the thigh, the leg, the foot). Therefore the cells can be prepared from vastus lateralis, vastus medialis, sartorius, biceps, triceps, quadriceps, the tensor of fascia lata, gastrocnemius, anterior tibialis, posterior tibialis, longus fibularis, common extensor of the toes, extensor of the fingers, peroneus, soleus, plantaris, gluteus maximus, rectus femoris, deltoid, latissimus dorsi, pectoralis, sternocleidomastoid, intercostal, homohyoid, rectus abdominis, psoas.

15 Said samples may be fresh samples and dissociated within less than 12h after harvesting. If desired or required, said samples may be kept under refrigeration or freezing until use. Therefore, the samples to be implemented in the process of the invention can be frozen samples (to be thawed before use). Muscle biopsies are then processed to obtain mononucleated cell suspensions, typically 1 to 96 hours after harvesting from a subject when

20 fresh or refrigerated biopsies are considered. Frozen muscle biopsies can be thawed and processed several years after harvesting. Muscle processing is well known in the art. Techniques for dissociation of a muscle tissue are known in the art. For example, the dissociation of a muscle tissue may comprise mincing the muscle tissue and digestion by enzymatic treatment of the minced muscle tissue, e.g., using collagenase (e.g., for 30 to 90

25 min at 37°C).—The cell suspension thereby obtained may then be filtered to collect the dissociated cells. Illustrative techniques are also described in WO 01/94555. These suspensions may be further used as such without any more preparation. Alternatively, they may be used after a step dedicated to the lysis of red cells, and/or after a purification step including the centrifugation on a Ficoll layer (see also below the specific use for enrichment

30 according to labeling under Figure 5). Alternatively, the suspensions may undergo a selection step according to methods known in the art, for example using fluorescence-based cell sorting, or immunomagnetic selection, or a combination of both. Alternatively, all the suspensions in any form above may be frozen until use.

By ALDH, it is herein intended aldehyde dehydrogenase (EC 1.2.1.3).

By ALDH⁺, ALDH⁺ or ALDH-positive, it is herein meant an aldehyde dehydrogenase presence or activity, more particularly an aldehyde dehydrogenase presence or activity that is superior to the one that would be detected on a negative control. In an assay for the detection of ALDH activity, a negative control could be performed i.e., under the same conditions but in the presence of an inhibitor of the aldehyde dehydrogenase activity under detection (e.g., 4-(diethylamino)benzaldehyde (DEAB), when ALDH activity is detected and/or measured). In particular, as provided below, cells having an ALDH activity are more particularly defined as BODIPY-AAA-stained cells, whose fluorescence is greater than that exhibited by at least 70%, preferably at least 80%, more preferably at least 90% of the cells exposed to DEAB.

The ALDH family is known to comprise at least 19 putatively functional genes (Vasiliou and Nebert, 2005). Each isoenzyme family is identified by an arabic number, e.g., ALDH1, ALDH2, ALDH3, ALDH4, ALDH5, ALDH6, ALDH7, ALDH8, ALDH9, ALDH16, ALDH18. Each family comprises several sub-families (which are identified by a letter; e.g., ALDH1A), optionally followed by an Arabic number (which denotes the individual gene within the sub-family; e.g., ALDH1A1).

ALDH⁺ cells enrichment may be performed by methods well known to those skilled in the art, a number of which is reviewed in EP2206774. For example, a person skilled in the art can use available anti-ALDH antibodies or produce anti-ALDH antibodies to detect the presence of ALDH. However, a preferred method implements the use of fluorescent synthetic substrates of ALDH, for example a fluorescent acetaldehyde, more particularly a fluorescent aminoacetaldehyde, the oxidation of which leads to fluorescent acetate or fluorescent aminoacetate, respectively, and cell sorting. Fluorescent synthetic aminoacetaldehyde (AAA) substrates (Jones et al.; Christ et al.) such as BODIPY-AAA (Aldefluor®) are retained intracellularly upon oxidation by ALDH, allowing the identification by flow cytometry and cell sorting of cell populations with low side scatter and high ALDH activity (SSC^{lo}/ALDH^{br}). The detection and relative quantification of ALDH enzymatic activity can be achieved rapidly and efficiently using this fluorescent substrate commercially available (Aldefluor®). The commercial kit comprises in particular a complex so-called "Aldefluor® buffer". However, in a particular embodiment incubation of the cells is carried out in a buffer comprising a calcium channel blocker such as Verapamil. The inventors have surprisingly observed that the survival

of the selected cells, but also their cardiogenic potential (in that they provide a better grafting) is higher when the buffer used for selecting the ALDH⁺ cells comprises such a calcium channel blocker. In a particular embodiment, the buffer comprises Phosphate Buffer Saline (PBS) supplemented with Fetal Bovine Serum (FBS), in particular 2% FBS, and a calcium channel blocker, for example Verapamil 0.5 to 500 μ M, in particular 1 μ M to 250 μ M, in particular 50 to 100 μ M, more particularly Verapamil 100 μ M. Accordingly, the invention also relates to a method for the identification or characterization of the ALDH activity of a skeletal muscle derived cell, comprising incubating said cell with a fluorescent acetaldehyde, more particularly a fluorescent aminoacetaldehyde, the oxidation of which leads to fluorescent acetate or fluorescent aminoacetate, in a buffer comprising Verapamil, and identifying or characterizing the cell with cell sorting methods. In a particular embodiment, the invention relates to a composition comprising Verapamil, in particular in a concentration as provided above. The composition may be a PBS buffer supplemented or not with FBS, for example with 2% FBS. In a particular embodiment, the cell population of the invention is an ALDH⁺ cell population, i.e. the cell population after selection comprises at least 50 %, preferably at least 60%, more preferably at least 70%, still more preferably at least 80%, still even more preferably at least 90%, most preferably at least 95%, still most preferably at least 98%, even still most preferably at least 99% of ALDH⁺ cells, for example 100% of ALDH-positive cells.

As mentioned above, optionally the cell population is selected after a step of purification, for example using a Ficoll or Percoll solution. This step can be useful for eliminating tissue debris from the skeletal muscle cell suspension. As such, the invention also relates to a method for the enrichment and selection of a skeletal muscle-derived cell population comprising **(a)** layering a skeletal muscle cell suspension onto a Ficoll solution, **(b)** centrifuging said solution, **(c)** harvesting living mononucleated cells at an interface where they form a collar, and **(d)** selecting and collecting ALDH⁺ cells from the harvested cells.

The cells are selected on the basis of ALDH expression or activity either with or without prior enrichment of muscle cells present in the source muscle sample. In a particular embodiment, ALDH⁺ cell selection is carried out without prior muscle cells enrichment from the muscle sample.

In a particular embodiment, the skeletal muscle derived cell population of the present invention is an ALDH⁺/CD34⁻ cell population. Illustrative means for CD34⁻ cell sorting are described in the examples.

- 5 The inventors have further characterized the features associated with the cell population according to the invention. In a particular embodiment, the cell population of the present invention, which is further characterized by at least 10%, in particular at least 20% of the cells in the ALDH⁺/CD34⁻ cell population, is associated with at least one of the following markers: CD140b, CD56, CD309, CD106, CD143, CD10, CD49a, CD49e, CD71, CD146, CD49c,
 10 CD140a, CD105, CD61, CD31, CD49f, CD47, CD44, CD29, CD36, CD9 and CD184. In another particular embodiment, at least 40%, in particular at least 50% of the cells in the ALDH⁺/CD34⁻ cell population is associated with at least one of the following markers: CD44, CD29, CD36, CD9, CD184 and CD47. It should be understood that any combination of the ALDH⁺/CD34⁻ markers with any one or more of the above CD markers is disclosed in the
 15 present application.

- In a further embodiment, the cell population of the present invention is an ALDH⁺/CD34⁻ cell population, with at least one, in particular all, additional feature selected from CD140b, CD56, CD309, CD106, CD143, CD10, CD49a, CD49e, CD71, CD146, CD49c, CD140a,
 20 CD105, CD61, CD31, CD49f, CD44, CD29, CD36, CD9 and CD184. In particular, the features are selected from CD9, CD10, CD44, CD49a, CD49e, CD56, CD106, CD140b and CD184. In a further particular embodiment, the features are selected from CD9, CD10 and CD184.

- 25 In a particular embodiment, the cell population of the present invention is an ALDH⁺/CD34⁻ cell population which comprises:
- at least 10% of CD140b-positive cells
 - at least 10% of CD56-positive cells
 - at least 10% of CD309-positive cells
 - 30 - at least 10% of CD106-positive cells
 - at least 10% of CD143-positive cells
 - at least 10% of CD10-positive cells
 - at least 10% of CD49a-positive cells
 - at least 10%, in particular at least 20% of CD49e-positive cells,

- at least 10%, in particular at least 20% of CD71-positive cells,
 - at least 10%, in particular at least 20% of CD146-positive cells,
 - at least 10%, in particular at least 20% of CD49c-positive cells,
 - at least 10%, in particular at least 20% of CD140a-positive cells,
 - 5 - at least 10%, in particular at least 20% of CD105-positive cells,
 - at least 10%, in particular at least 20% of CD61-positive cells,
 - at least 10%, in particular at least 20% of CD31-positive cells,
 - at least 10%, in particular at least 20% of CD49f-positive cells,
 - at least 40%, in particular at least 50% of CD44-positive cells,
 - 10 - at least 40%, in particular at least 50% of CD29-positive cells,
 - at least 40%, in particular at least 50% of CD36-positive cells,
 - at least 40%, in particular at least 50% of CD9-positive cells, or
 - at least 40%, in particular at least 50% of CD184-positive cells.
- 15 In a further embodiment, the invention relates to a skeletal muscle-derived cell population selected on the basis of the expression of ALDH, the absence of CD34 and the presence of at least one, in particular all, of the markers selected in the group consisting of CD140b, CD56, CD309, CD106, CD143, CD10, CD49a, CD49e, CD71, CD146, CD49c, CD140a, CD105, CD61, CD31, CD49f, CD44, CD29, CD36, CD9 and CD184. It should be understood that the
- 20 cell population according to the invention is defined in relation to expression of CD markers identified in said population. However, it cannot be excluded that one or more of these CD markers are not associated with the cell having the cardiogenic capacity.
- The skeletal muscle-derived cell population of the invention may be processed to obtain a cell
- 25 population having cardiogenic differentiation capacities. By "a cell population having cardiogenic capacities" or "a cell population having cardiogenic differentiation abilities", it is herein meant a cell population able to form bona fide cardiac tissue *in vivo* after cell transplantation. Said cells express cardiac proteins such as alpha-actinin, cardiac troponin that can be observed using immunohistological techniques. At early stages the expression of
- 30 cardiac differentiation factors such as Islet-1, and/or Nkx2.5 and/or GATA-4 can be observed using immunohistological techniques. In a preferred embodiment, at late stages of differentiation, these cells express also connexin-43.

The invention also relates to method for the production of a cell population having cardiogenic differentiation capacities, comprising providing a skeletal muscle-derived cell population as defined herein above, and culturing said population *in vitro*, thereby obtaining a cell population having cardiomyogenic differentiation abilities. The invention further relates to a skeletal muscle-derived cell population having cardiogenic differentiation abilities, wherein said cell population is obtainable by a method as defined in this paragraph.

In a particular embodiment, the skeletal muscle-derived cell population is an ALDH⁺ skeletal muscle-derived cell population, more particularly an ALDH⁺/CD34⁻ cell population. After sorting, the skeletal muscle derived cell population may be collected in an appropriate medium and stored at 4°C as long as 24 hours. In a particular embodiment, the skeletal muscle-derived cell population may be cultured *in vitro* in a cell culture medium, in particular a muscle cell medium, more particularly a skeletal muscle cell culture medium. The medium may advantageously be supplemented with differentiation and/or growth factors immediately, or substantially immediately (e.g. for example not more than 20 minutes) after placing the cells in the culture medium after the refrigeration step. Representative factors include, for example, Bone Morphogenetic proteins (BMPs) such as BMP-2 and BMP-4, IGF-1 and bFGF. In a particular embodiment, the medium does not include retinoic acid. In a particular embodiment of the method, the culture medium is supplemented with BMP-2, BMP-4, IGF-1 and/or b-FGF. In a further particular embodiment, the culture medium is supplemented with BMP-2, BMP-4 and IGF-1 and optionally, but preferably, with bFGF. The skeletal muscle-derived cell population is cultured *in vitro* for a period of time sufficient to produce cardiogenic cells. For example, the cells may be cultured for at least about 24 hours, about 30 hours, about 36 hours, about 42 hours, about 48 hours. In a particular embodiment of the invention, the cells are cultured *in vitro* between about 24 hours and about 20 days, in particular between 24 hours and 15 days. The cells are for example cultured during between 48 hours and 10 days. In a particular embodiment, the culture medium is supplemented frequently with differentiation and/or growth factors as described above, or replaced frequently with fresh culture medium supplemented with these differentiation and/or growth factors, in particular each 24 hours. Cardiogenic cells express Nkx2.5, GATA4, Islet1, alpha actinin, cardiac troponin at early stages after addition of the differentiation and/or growth factors. At later stages after addition of differentiation and/or growth factors, cardiogenic cells express connexin-43. After the *in vitro* culture step, the skeletal muscle-derived cardiogenic cell population may be used directly for transplantation or frozen prior to administration to a

subject in need thereof. After thawing, the cells are cultured in a skeletal muscle cell culture medium, preferably supplemented with differentiation and/or growth factors as described above.

5 A further object of the invention corresponds to a cardiogenic cell population derived from a skeletal muscle-derived cell population as provided above. This cell population may be otherwise referred to as a cell population having bona fide cardiogenic capacities. In particular, the cardiogenic cell population is obtainable according to the method described in the previous paragraph. In particular, the invention relates to a cardiogenic cell population
10 having the ability to form cardiac tissue *in vivo* after transplantation, said cardiogenic population being obtainable by *in vitro* culturing a skeletal muscle-derived cell population (preferably an ALDH⁺/CD34⁻ skeletal muscle-derived cell population). Particular embodiments of the method for obtaining the cardiogenic cell population of the invention from skeletal muscle are provided in the previous paragraph and throughout this application,
15 in particular in the examples.

Another object of the invention relates to a composition useful for committing *in vitro* a cell population having cardiogenic capacities. In particular, the composition comprises one or more differentiation and/or growth factors such as BMP-2, BMP-4, IGF-1 and bFGF. In
20 particular, the composition according to the invention comprises BMP-2, BMP-4 and IGF-1, and optionally bFGF. In a particular embodiment, the composition according to the invention is a cell culture medium (e.g. a muscle cell culture medium) supplemented with BMP-2, BMP-4, IGF-1 and/or bFGF, in particular a medium supplemented with BMP-2, BMP-4 and IGF-1, and optionally with bFGF. In a preferred embodiment, the medium is supplemented
25 with BMP-2, BMP-4, IGF-1, and bFGF.

Any concentration of BMP-2 or BMP-4 can be used. For example, between 1 and 50 ng of BMP-2 or BMP-4 per ml (e.g., about 10 ng of BMP-4 and 10 ng of BMP-2 per ml) can be used. IGF-1 can be any polypeptide having IGF-1 activity, such as human IGF-1. For
30 example, IGF-1 can be recombinant IGF-1 or synthetic IGF-1. Any concentration of IGF-1 can be used. For example, between 1 to 100 ng of IGF-1 per ml (e.g., about 10 ng of IGF-1 per ml) can be used. bFGF can be any polypeptide having bFGF activity, such as human bFGF. For example, bFGF can be recombinant bFGF or synthetic bFGF. Any concentration

of bFGF can be used. For example, between 1 to 100 ng of b-FGF per ml (e.g., about 10 ng of bFGF per ml) can be used.

The invention also relates to a method for producing cells for transplantation into myocardial tissue of a mammal comprising the steps of: (a) culturing skeletal muscle-derived

5 ALDH⁺/CD34⁻ cells in a culture medium containing differentiation and/or growth factors such as b-FGF, BMP-2, BMP-4 and IGF-1 to induce said cells to commit into cardiogenic cells; and (b) collecting the committed cells of step (b). In a particular embodiment, the method for producing cells for transplantation into myocardial tissue of a mammal comprises the steps of:

10 (a) selecting ALDH⁺ cells from a skeletal muscle tissue in a buffer containing a calcium channel blocker such as Verapamil; (b) further selecting CD34⁻ cells from the cells of step (a); (c) culturing the cells from step (b) in a culture medium containing differentiation and/or growth factors such as b-FGF, BMP-2, BMP-4 and IGF-1 to induce said cells to commit into cardiogenic cells; and (d) collecting the committed cells of step (c). In a particularly preferred

15 embodiment, the method comprises:

(a) selecting ALDH⁺ cells from a skeletal muscle tissue in a buffer containing Verapamil at a concentration comprised between 50 and 100 μ M;

(b) further selecting CD34⁻ cells from the cells of step (a);

(c) culturing the cells from step (b) in a culture medium containing b-FGF, BMP-2, BMP-4

20 and IGF-1 to induce said cells to commit into cardiogenic cells; and

(d) collecting the committed cells of step (c)

Uses of the cardiogenic skeletal muscle-derived cell population

25 The invention relates to a cardiogenic, skeletal muscle-derived cell population as described above for use in the treatment of a cardiac defect, disease or pathology. Said cells may in particular be used for cell therapy of degenerative diseases. The invention also relates to the use of such a cell population for the manufacture of a medicament for use in the treatment of a cardiac defect, disease or pathology. In addition, the invention relates to a method for the

30 treatment of a cardiac defect, disease or pathology, comprising administering to a subject in need thereof a cardiogenic, skeletal muscle-derived cell population according to the invention.

In one embodiment, the cardiogenic skeletal muscle-derived cell population is introduced or transplanted in the heart of the subject. Several modalities exist and are available to persons

with the skills in the art. The cells can be injected directly, using a classical or a specific needle, inside the portion of myocardial tissue to be treated, under view control, following sternotomy or thoracotomy. The cells can be injected directly through a trans-thoracic approach using a dedicated needle and under control of a visualization system, such as echocardiography. The cells can be injected using catheters, and through a venous or an arterial route. Using catheters, cells can be delivered in the coronary artery. Alternatively, using catheters equipped with a thin needle, cells can be delivered in the endocardial wall, after the catheter has reached the heart cavity. Also, using catheters equipped with a thin needle, cells can be delivered in the myocardial tissue, after the catheter has progressed along the coronary veins. Alternatively, the cells can be injected in the systemic circulation through an arterial or a venous route. Alternatively, the cells can be administered in the form of cellular sheets composed of one or several layers of cells containing one or up to several different categories of cells, in which case the cellular sheets are fixed to the myocardial tissue. Alternatively, cells can be administered in the form of cellular patches consisting of one or several categories of cells, mixed with one or several substrates, in which case the cellular patches are fixed to the myocardial tissue. The nature of the substrates may vary to confer them viscosity, rigidity or stiffness, and they may be supplemented with cytokines to promote the survival, the integration, the migration, the proliferation or the differentiation of cells mixed with these substrates. The present invention encompasses both single and multiple administrations of the cell population, either at a single site or multiple sites of the acceptor tissue.

As provided above, the skeletal muscle sample is preferably obtained from an autologous or heterologous (xenogenic or allogenic source) human or animal source. Preferably, the skeletal muscle sample is from an allogenic or autologous source, preferably autologous.

The cardiogenic cell population (i.e. a skeletal muscle-derived cell population that has been cultured *in vitro* as provided above) is administered to the subject in need thereof in the form of a suspension of mononucleated cells in a pharmaceutically or physiologically acceptable carrier, excipient or diluent. The compositions of the present invention may further comprise at least one pharmaceutically and/or physiologically acceptable vehicle, such as at least one diluent, excipient, additive, pH adjuster, emulsifier or dispersing agent, pH buffering agents, preservative, surfactant, gelling agent, as well as buffering and other stabilizing and solubilizing agent, etc. Appropriate pharmaceutically acceptable vehicles and formulations

include all known³⁰ pharmaceutically acceptable vehicles and formulations, such as those described in "Remington: The Science and Practice of Pharmacy", 20th edition, Mack Publishing Co.; and "Pharmaceutical Dosage Forms and Drug Delivery Systems", Ansel, Popovich and Allen Jr., Lippincott Williams and Wilkins. In general, the nature of the vehicle

5 will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise, in addition to the one or more contrast agents, injectable fluids that include pharmaceutically and physiologically acceptable fluids, including water, physiological saline, balanced salt solutions, buffers, aqueous dextrose, glycerol, ethanol, sesame oil, combinations thereof, or the like as a vehicle. The medium also may contain

10 conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. The carrier and composition can be sterile, and the formulation suits the mode of administration. A composition or therapy product of the invention can for example be a liquid solution, suspension or emulsion. The composition can be formulated with traditional binders and

15 carriers, such as triglycerides. The invention thus further relates to a composition comprising, in a pharmaceutically or physiologically acceptable carrier, excipient or diluent, a cardiogenic skeletal muscle-derived cell population as described above. The composition can be prepared by resuspending the cells in a suitable liquid or solution such as sterile physiological saline or other pharmaceutically or physiologically acceptable injectable aqueous liquids. The amounts

20 of the components to be used in such compositions can be routinely determined by those having skill in the art.

To optimize transplant success, the closest possible immunological match between the donor and the recipient is desired. Thus, the preferred embodiment involves an autologous

25 transplant. However, if an autologous source is not available (for example, if the subject has a genetic defect incompatible with transplanting its own cells for cardiac transplant), donor and recipient class I and class II histocompatibility antigens can be analyzed to determine the closest match available. This minimizes or eliminates immune rejection and reduces the need for immunosuppressive or immunomodulatory therapy. However, if required,

30 immunosuppressive or immunomodulatory therapy can be started before, during and/or after the transplant procedure. A skilled practitioner can modulate the nature and posology of immunosuppressive regimen to set up and follow in each individual situation.

The number of cells of the present invention administered and the mode of administration may vary depending on the site and condition being treated. For example, 10^6 to 10^9 or more cells may be administered to the subject.

5 A skilled practitioner can modulate the amounts and methods of cell-based treatments according to requirements, limitations, and/or optimizations determined for each case.

The present invention embraces the use of the cardiogenic skeletal muscle-derived cell population as defined above for treating a cardiac defect, disease or pathology. The terms "cardiac defect, disease or pathology" notably include any extrinsic cardiomyopathy and/or an
10 intrinsic cardiomyopathy, including any disorder which may require heart transplantation. Particular extrinsic cardiomyopathy notably comprise coronary artery disease, some congenital heart diseases, nutritional diseases affecting the heart, ischemic cardiomyopathy, hypertensive cardiomyopathy, valvular cardiomyopathy, inflammatory cardiomyopathy, cardiomyopathy secondary to a systemic metabolic disease, alcoholic cardiomyopathy,
15 diabetic cardiomyopathy; preferably coronary artery disease, congenital heart disease, ischemic cardiomyopathy, hypertensive cardiomyopathy, valvular cardiomyopathy. Particular intrinsic cardiomyopathy notably comprise dilated cardiomyopathy, hypertrophic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, restrictive cardiomyopathy including the obliterative cardiomyopathy, noncompaction cardiomyopathy;
20 preferably dilated cardiomyopathy; some congenital heart diseases, such as the Fallot's tetralogy.

The cardiogenic skeletal muscle-derived cell population can be used as a vector, for the delivery of genes, proteins or substances in a therapeutic perspective. Depending on the
25 therapeutic indication, cells may be used as vectors without modification. For example, to repair local myocardial damages, autologous cells may be used without modification (Menasché et al.; 2008). Alternatively, cells may be engineered to express proteins or factors involved in myocardial healing, vascularization or regeneration (Von Degenfeld et al.). Alternatively, in the context of myocardial diseases of genetic origin, the cardiogenic skeletal
30 muscle-derived cell populations of the invention can be genetically engineered to allow the correction of the effect of the pathological mutation (Ousterout et al.; Quenneville et al.).

The cardiogenic skeletal muscle-derived cell population can be therefore genetically engineered under these circumstances, for example for gene edition, and/or gene repair, and/or gene replacement, and/or for gene addition and/or suppression, before being

administered to the human or non-human animal. Alternatively, the cell populations of the invention can be genetically engineered, as described above, before being cultured for proliferation and subsequently used for administration to a human or non-human animal. According to a particular embodiment, the cell population is genetically engineered after
5 treatment with the composition useful for committing *in vitro* a cell population having cardiogenic capacities as described above, and then either used directly or expanded before use.

Other uses of the cell population according to the invention

10 The invention also relates to a method for screening several classes of compounds. Such compounds may be able to induce cardiogenic differentiation (e.g. bone morphogenetic proteins, transforming growth factors, vascular endothelial growth factors, interleukins, cardiotrophin1, retinoic acid...), and/or to modify the function of cardiac cells (e.g. beta-blockers, adrenergic agents...), and/or to analyze the function of cardiac cells (e.g. channel
15 blockers...). Such screenings comprise contacting a test compound with a cell population according to the invention, and determining the cardiogenic commitment, differentiation, fate, behavior of said population.

The invention also relates to a method for screening genetic mutations within cells of patients
20 or animal models (e.g. familial dilated cardiomyopathy, Duchenne muscular dystrophy, Emery-Dreyfus muscular dystrophy...). Such screening comprise extracting and selecting skeletal muscle cells from patients presenting with cardiac pathologies or diseases, coaxing them toward cardiogenesis using the cardiac differentiation medium as provided above, expand them in numbers compatible with biochemical analysis, extracting and analyzing their
25 molecular content and expression (DNA, RNA, proteins) using methodologies known from persons skilled in the art.

Alternatively, cardiogenic skeletal muscle-derived cells may be immortalized, before or after the induction process, using methodologies known in the art, for example as described by Mamchaoui et al., thus providing an extended source of cells useful for studying cell biology,
30 cell physiology, and to develop screening applications as mentioned above.

EXAMPLES

Materials and Methods

The cells were extracted from muscle biopsies, and several animal species can be considered as providers for these cells.

5

Human muscle biopsies

Frozen or fresh human muscle biopsies were obtained in agreement with the French Regulatory Health Authorities and our Ethics Committee via the Tissue Bank for Research of the French Association against Myopathies (MYOBANK). Muscle samples were obtained as
10 a *res nullus* during orthopedic surgery with signed informed consent of the patients. The weight of muscle samples obtained varied from less than 50 mg to several grams. The muscle biopsies were transported and preserved in a synthetic medium supplemented with antibiotics. Typically, but not exclusively, such a medium can consist in DMEM supplemented with gentamycin. The muscle biopsies were processed for cell extraction (see below) 1 to 96 hours
15 after harvesting from the patients, i.e. they could be stored for 1 to 96 hours before processing.

Macaque muscle biopsies

Fresh skeletal and cardiac muscle biopsies were obtained from *Macaca Fascicularis* or
20 *Macaca Mulatta* at the time of sacrifice of the animals. The weight of muscle samples varied from less than 1 g to several grams. For commodity reasons and because of the availability of material, the muscle processing was performed 2 to 24 hours after sample collection. Macaque muscle biopsies, however, can be kept in transport medium, such as (but not limited to) DMEM supplemented with gentamycin for 1 to 96 hours.

25

Mouse muscle biopsies

Fresh mouse muscle biopsies were obtained from 8-56 week old animals. C57BL/6 mice were purchased from Janvier CERJ. Mice were maintained in our facilities under specific pathogen-free conditions. All experimental procedures were performed on female age-
30 matched mice according to the guide for the care and use of laboratory animals published by the NIH (publication No. 85-23, revised 1996). Following deep anesthesia using ketamine and xylazine, animals were sacrificed by cervical dislocation and the muscles of their upper and lower limbs were harvested and pooled. The muscle processing was performed 1 to 24 hours after sample collection.

Muscle processing

Muscle biopsies were sliced using scissors or blades. Then, muscle fragments were finely minced using scissors. The minced homogenate was digested using enzymes. Typically, but not in a limitative manner, the digestion was processed for 1 hour at 37°C using 0.2% type II collagenase (Worthington) dissolved in a medium containing DMEM supplemented with 10% fetal bovine serum and gentamycin (25µg/ml). The duration of incubation, the nature of collagenase and its concentration, the composition of the enzymatic blend may be changed and adapted to individual situations. Mechanical dissociation was completed by passage of the cell dissociate through a pipette, and/or a needle. As a non-limitative example, a 10ml pipette can be used, and an 18G needle. The suspension was filtrated through one or a series of strainers. Typically, 100 then 40µm cell strainers (Becton-Dickinson) were used. The resulting cell suspensions were centrifuged, washed, and used directly or frozen for later use. A medium used for cell freezing typically contains 70% DMEM, 20% FBS, 10% DMSO, and gentamycin.

Characterization and quantification of ALDH-expressing cells using Aldefluor reagent and cytofluorimetry

Fresh or frozen cells were used. Frozen cells were thawed rapidly in water bath, centrifuged and washed in PBS containing 2% FBS. Fresh cells were washed in PBS containing 2% FBS. Following centrifugation, fresh cells or frozen cells were incubated in Aldefluor® assay buffer, or an alternative buffer (see below) containing the ALDH substrate (1µM), for 20 to 45 min, at 37°C (Stemcell Technologies). Alternatively, the concentration of substrate and the duration of incubation may be changed or adapted to different situations. As an example, human cells undergo a 20 min incubation. Mouse cells undergo a 45 min incubation. Canine cells undergo a 30 min incubation. Controls were obtained by prior incubation of cells with 50mM of the specific ALDH inhibitor DEAB. Cells were centrifuged, suspended in Aldefluor® kit buffer. Then cells were labeled with antibodies directed against extracellular markers (see a typical list under Table 1). FACS settings for analysis of ALDH expression are presented in Table 2. Cells were analyzed by flow fluorocytometry (FacsCalibur, BD) using the Cell Quest Software. Alternatively, other FACS machines can be used. Aldefluor ® labeling was also used for cell sorting in combination with labeling of extracellular markers (see below).

Table 1

CD / alternate name(s) / (function)	Clone #	Isotype	Format	Cat. #	Provider
CD9 / TSPAN29 / (cell adhesion and migration)	M-L13	Ms IgG ₁ , κ	PE	555372	BD Pharmingen™
CD10 / CALLA / (neutral endopeptidase)	HI10a	Ms IgG ₁ , κ	PE	555375	BD Pharmingen™
CD29 / ITGB1 / (cell adhesion)	MAR4	Ms IgG ₁ , κ	PE	555443	BD Pharmingen™
CD31 / PECAM-1 / (component of endothelial cell intercellular junctions)	WM59	Ms IgG ₁ , κ	PE	555446	BD Pharmingen™
CD34 / gp105-120, Sialomucin / (cell-cell adhesion factor)	581	Ms IgG ₁ , κ	APC	555824	BD Pharmingen™
CD36 / FAT / (receptor for selective cholesteryl ester uptake)	CB38	Ms IgM, κ	PE	555455	BD Pharmingen™
CD44 / HCELL / (cell adhesion and migration)	515	Ms IgM, κ	PE	550989	BD Pharmingen™
CD45 / LCA / (tyrosine phosphatase)	HI30	Ms IgG ₁ , κ	APC	555485	BD Pharmingen™
CD47 / (involved in the increase in intracellular calcium concentration)	B6H12	Ms IgG ₁ , κ	PE	556046	BD Pharmingen™
CD49a / α1 integrin / (cell-surface receptor for collagen and laminin)	SR84	Ms IgG ₁ , κ	PE	559596	BD Pharmingen™
CD49b / α2 integrin	DX5	Ms IgM, κ	PE	553858	BD Pharmingen™
CD49c / α3 integrin	C3 IL1	Ms IgG ₁ , κ	PE	556025	BD Pharmingen™
CD49d / α4 integrin	L25	Ms IgG _{2b} , κ	PE	340976	BD Pharmingen™
CD49e / α5 integrin	C3 IL1	Ms IgG ₁ , κ	PE	555617	BD Pharmingen™
CD49f / α6 integrin	GoH3	Ms IgG ₁ , κ	PE	555736	BD Pharmingen™
CD56 / NCAM / (cell-cell adhesion)	My31	Ms IgG ₁ , κ	PE	345810	BD Pharmingen™
CD61 / β3 integrin	VI-PL2	Ms IgG ₁ , κ	PE	555754	BD Pharmingen™
CD71 / Transferrin receptor protein 1	M-A712	Ms IgG _{2a} , κ	PE	555537	BD Pharmingen™
CD105 / Endoglin / (part of the TGF beta receptor complex)	166707	Ms IgG ₁ , κ	PE	FAB10971P	R&D Systemsä
CD106 / Vascular cell adhesion protein 1 / (cell adhesion)	51-10C9	Ms IgG ₁ , κ	PE	555647	BD Pharmingen™
CD133	AC133	Ms IgG ₁ , κ	PE	130-080-801	Miltenyi™
CD140a / PDGF Receptor a	αR1	Ms IgG _{2a} , κ	PE	556002	BD Pharmingen™
CD140b // PDGF Receptor b	28D4	Ms IgG _{2a} , κ	PE	558821	BD Pharmingen™
CD143 / Angiotensin-converting enzyme	BB9	Ms IgG ₁ , κ	PE	557928	BD Pharmingen™
CD146 / MCAM / (marker for endothelial cell lineage)	P1H12	Ms IgG ₁ , κ	PE	550315	BD Pharmingen™
CD172a / b (SIRPα/β)	SE5A5	Ms IgG ₁ , κ	APC		Biolegend™
CD184 / CXCR4 / (receptor specific for stromal-derived-factor-1 SDF-1)	12G5	Ms IgG _{2a} , κ	PE	555974	BD Pharmingen™
CD309 / Flk-1/ (vascular endothelial growth factor receptor 2)	89106	Ms IgG ₁ , κ	PE	FAB357P	R&D Systemsä
Mouse IgG ₁ , κ	MOPC-21	Ms IgG ₁ , κ	PE	555749	BD Pharmingen™
Mouse IgG ₁ , κ	MOPC-21	Ms IgG ₁ , κ	APC	555751	BD Pharmingen™
Mouse IgG _{2a}	X39	Ms IgG _{2a} , κ	PE	349053	BD Pharmingen™
Mouse IgG _{2b} , κ	27-35	Ms IgG _{2b} , κ	PE	555743	BD Pharmingen™
Mouse IgM, κ	G155-228	Ms IgM, κ	PE	555584	BD Pharmingen™

Table 2

5 Cytometer Type: FACSCalibur BD Pharmingen

Detectors/Amps:

Param	Detector	Voltage	Amp Gain	Mode
P1	FSC	E00	1.27	Lin
P2	SSC	357	5.02	Lin
P3	FL1	355	1.00	Log
P4	FL2	356	1.00	Log
P5	FL3	635	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL4	480		Log

10 Threshold

Primary Parameter: FSC

Value: 0

Secondary Parameter: None

15

Compensation

FL1-	2.6%	FL2
FL2-	12.0%	FL1
FL2-	0.0%	FL3
FL3-	0.0%	FL2
FL3-	0.0%	FL4
FL4-	0.0%	FL3

Alternative preparation of cells using Ficoll

Alternatively, cells could be layered onto a Ficoll solution, so as to separate the tissue debris
 20 from the mononucleated living cells.

Muscle cells from 5 Macaques aged 5-20 year were enzymatically-dissociated prior centrifugation onto a Ficoll cushion, whose density is superior to that of the physiological medium. Briefly, enzymatically-dissociated cells were suspended in PBS medium and slowly added on top of an equal volume of Histopaque (Sigma). This preparation was centrifuged at 300g, 15 minutes, 20°C. Living mononucleated cells were concentrated in a ring at the interface on the top of the Ficoll layer, where they formed a collar. They were harvested using a thin pipette, transferred in a new tube, then washed in a physiological buffer, centrifuged and suspended in a buffer appropriate for further use (i.e. one buffer adapted to the measurement of ALDH activity, or a buffer adapted to phenotyping, to cell sorting, to cell culture, or to cell transplantation).

Alternative incubation in a custom-made buffer

Alternatively, incubations of the cells in view of detection of ALDH activity using the Aldefluor reagent, and eventually cell sorting, could be performed in a buffer made of PBS, supplemented with 2% FBS and 100µM Verapamil, instead of the complex, commercially-available so-called “Aldefluor buffer”.

Phenotyping of cell populations using antibodies directed against extracellular markers

Cells were centrifuged and suspended in Aldefluor Buffer or alternative custom-made buffer as described above. They were incubated in presence of antibodies directed against specific markers (see Table 1). Extracellular markers were detected by incubations with allophycocyanin (APC)-conjugated antibodies (CD34, CD45, Becton-Dickinson), or Phycoerythrin (PE)-conjugated antibodies (CD9, CD10, CD29, CD31, CD36, CD44, CD47, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD56, CD61, CD71, CD105, CD106, CD133, CD140a, CD140b, CD143, CD146, CD172a, CD184, CD309). These antibodies are listed in Table 1 and were used at 1/30 (15 minutes, 4 °C). Non-specific fluorescence was determined using negative isotype controls (BD). Cells were centrifuged, suspended in Aldefluor kit buffer or alternative custom made buffer, and analyzed by flow fluorocytometry (FacsCalibur, Becton-Dickinson) using the Cell Quest Software. Other FACS machines and softwares can be used.

Cell sorting

Aldefluor® labeling was used for sorting cells, eventually in combination with labeling of extracellular markers. The settings are presented in Table 2.

Following incubation in presence of Aldefluor reagent, then incubation in presence of an antibody directed against a species-specific CD34 antigen (see above), cells were sorted on the basis of both ALDH activity and expression of CD34 using a Vantage SE DiVA (Becton Dickinson). Anti-CD34 antibodies (all from BD) were produced by different clones: 581 for Human cells, 563 for non-human primate cells, RAM34 for mouse cells.

Following incubation in presence of Aldefluor reagent (see above), the cells from murine models were sorted on the basis of ALDH activity.

Cell cultures before transplantation

Sorted cells were centrifuged, washed and seeded in 6-wells, 12-wells or 24-wells plates depending on the initial number of sorted cells. The cells were induced into the cardiac lineage in a proliferation medium that typically contained 80% modified synthetic custom-made MCDB medium (Hyclone), 20% fetal bovine serum (Hyclone), Gentamycin (25µg/ml), and 10 ng/ml of each following cytokines: human recombinant bFGF (R&D systems, Lille, France); human recombinant BMP-2 (Sigma), human recombinant BMP-4 (R&D systems) and human recombinant IGF-1 (R&D systems).

Alternatively, the synthetic MCDB medium may be replaced by other synthetic culture media containing salts, amino-acids, nutriment. The concentration of serum may be adapted by reducing or increasing it. The antibiotic may be replaced by other antibiotics or even omitted.

The cytokines assembled in the cocktail may be human recombinant, or may be recombinant proteins prepared on the background of other animal species (e.g. mouse, dog, monkey, rat...).

The medium was changed every day. Alternatively, the medium may be changed every other day. The cultures were grown for 1 to 6 days, then cells were harvested by trypsinization and directly used for transplantation studies. Alternatively, cells could be obtained without the use of trypsin enzyme, e.g. cells could be detached using substrates sensitive to culture conditions, or using non-tryptic enzymes, or using mechanical means.

Flow cytometry Analysis of differentiating cells

At various times after setting of the cultures (24 hours, 48 hours and 72 hours), the cell layers were washed in PBS, fixed in paraformaldehyde 4% in PBS (PFA 4%) 10 min at room temperature, permeabilized using triton (0.2%) 10 min at room temperature, rinsed in PBS and incubated using one antibody directed against a transcriptional factor specifically involved in cardiac differentiation such as Islet-1, Nkx2.5 and GATA-4. Cells were first

incubated with the primary Rabbit antibody (Islet-1, Nkx2.5 or GATA-4, 1/50, 90 min at 4°C) followed by the secondary Goat anti-Rabbit antibody linked to a fluorophore (FITC, 1/100 in PBS, 30 min at 4°C) and analyzed by FACS as above. The three combinations are summarized in Table 3.

5

Table 3:

					Combination		
Primary Antibodies	Poly/monoclonal	Clone	Host	Reactivity	1	2	3
Anti-Islet-1	polyclonal	ND	Rabbit	Human, Mouse			x
Anti-Nkx2.5	polyclonal	H-114			x		
Anti-GATA-4	polyclonal	H-112				x	
Secondary antibodies							
Goat to Rabbit IgG FITC	polyclonal		Goat	Rabbit	x	x	x

Cytofluorescence analysis of differentiating cell cultures

10

Seven days after setting of the cultures, the cell layers were washed in PBS, fixed using PFA 4% and permeabilized with methanol/acetone 10 min at room temperature. Then, cells were incubated at room temperature for 1h using two different panels of antibodies and reagents that may be directed against membrane (connexin-43), cytoplasmic (actinin, troponin), or nuclear (DAPI) structures. The combinations are summarized in the following table. Cells were mounted with Vectashield-DAPI. Negative controls were obtained by substitution of the primary Ab with DFBS. Cells were observed using an inverted microscope equipped with fluorescence (Olympus Optical Co).

15

20 Table 4

Primary Antibodies (1/400, 1h)	Poly/monoclonal	Clone	Host	Reactivity	Combination	
					1	2
Anti-alpha-actinin (IgG1)	monoclonal	EA-53	Mouse	Human, Mouse	x	
Anti-Cardiac-Troponin I (IgG2b)		284(19C7)				x
Anti-Connexin 43 IgG(H+L)	polyclonal		Rabbit		x	x
Secondary antibodies (1/400, 1h)						
Goat to Rabbit IgG(H+L) Alexa Fluor 488	polyclonal		Goat	Rabbit	x	
Goat to Rabbit IgG(H+L) Alexa Fluor 568						x
Goat to Mouse IgG1 Alexa Fluor 568				Mouse	x	
Goat to Mouse IgG2b Alexa Fluor 488						x
DAPI (350)					x	x

Preparation of cells and their transplantation into mouse myocardium

The cells treated for 48 to 72h as above were harvested using trypsin-EDTA (0.025%, Invitrogen), suspended in PBS supplemented with 0.5% bovine serum albumin (BSA, Sigma) and kept on ice until injection. All experimental procedures were performed on female age-matched mice according to the guide for the care and use of laboratory animals published by the NIH (publication No. 85-23, revised 1996). Immunodeficient animals (NOD-SCID mice) were anaesthetized using ketamine (75 mg/kg) and xylazine (15 mg/kg) and ventilated (MiniVent, Type845; Hugo Sachs Elektronik, Germany). Upon small left thoracotomy at the level of the 5th intercostal space, 10 000 to 150 000 treated cells were injected at four sites on the anterior-lateral wall of the left ventricle under a total volume of 40µl, using a Hamilton syringe and a 32G needle

Histological characterization of cell integration

The animals were sacrificed 4wks after transplantation under general anesthesia. Hearts were snap frozen in liquid-nitrogen-cooled isopentane and serial cryosections (7 µm) collected in a transverse orientation from apex to basis, in order to ensure a complete overview of cell distribution. Implantation of human cells in host muscles was evaluated by species-specific labeling of the human protein lamin A/C (not staining the mouse antigen). Connexin 43 staining delineated the membrane of cardiomyocytes. Cardiac troponin I and α -actinin stained the cytoplasm of cardiomyocytes. Fast skeletal myosin heavy chain (MHC) stained skeletal muscle fibers and not the cardiac cells. The combinations are summarized in the following table. Antibodies were used diluted 1/400 in PBS and incubated for 1h at room temperature. Cells were mounted with Vectashield containing DAPI. Negative controls were obtained by substitution of the primary Ab with fetal bovine serum. Cells were observed using an inverted microscope equipped with fluorescence (Olympus Optical Co).

Table 5

Primary Antibodies (1/400, 1h)	Poly/monoclonal	Clone	Host	Reactivity	Combination	
					1	2
Anti-alpha-actinin (IgG1)	monoclonal	EA-53	Mouse	Human, Mouse	x	
Anti-Cardiac-Troponin I (IgG2b)		284(19C7)				x
Anti-Connexin-43 IgG(H+L)	polyclonal		Rabbit		x	x
Anti-Lamin A/C (IgG2b)	monoclonal	636	Mouse	Human	x	

Anti-Myosin Heavy Chain (IgG1)		MY-32	Mouse	Human, Mouse		x
Secondary antibodies (1/400, 1h)						
Goat to Rabbit IgG(H+L) Alexa Fluor 350	polyclonal		Goat	Rabbit	x	x
Goat to Mouse IgG1 Alexa Fluor 568				Mouse	x	x
Goat to Mouse IgG2b Alexa Fluor 488					x	x

Results

5 *Extraction of cells from muscle biopsies*

The mechanical and enzymatic dissociation of muscles tissues allowed the preparation of mononucleated cell suspensions. These suspensions could be further used as such without any more preparation. They could be used after a step dedicated to the lysis of red cells, or after a purification step including the centrifugation on a Ficoll layer (see also below the specific use for enrichment according to labeling under Figure 5), or after a round of freezing and thawing procedures.

Tables of antibodies and FACS settings

The cells were characterized using one or several markers, alone or in association. The cells could be selected and the population enriched using a combination of the techniques above and/or the use of one or several markers. As a non limitative example, the marker list is provided in Table 1. The settings of cytofluorimeter are provided in Table 2 as an example.

Phenotypical characterization using CD markers

20 The characterization of Human skeletal muscle cells is summarized in Figure 1. The vertical axis represents the SSC (Side scatter, i.e. the granularity of the cells) and the horizontal axis represents the marker under scrutiny (CD). Log scales are used. The proportions of cells expressing a given marker were a mean of 7 evaluations +/- SD.

Populations were expressing the following markers, in ascending order:

25 CD106 < CD140a = CD309 < CD71 < CD140b = CD49c < CD61 < CD45 = CD143 < CD105 < CD184 < CD44 = CD49a < CD146 < CD49f < CD10 < CD56 < CD29 < CD9 < CD49e < CD31 = CD36 < CD34 < CD47.

Analysis of ALDH expression and characterization of populations

The mechanical and enzymatic dissociation of cardiac and skeletal muscle tissues provided cell suspensions. ALDH was chosen as one of the markers associated to the cell populations extracted from muscles. ALDH is a functional marker, which depends on cellular activity and viability. The preparations contained quantifiable proportions of cells expressing ALDH, as observed *in vitro* using the Aldefluor reagent and presented on Figure 2. These results open the way to the exploration of the role and regulation of these cell types in normal or pathological contexts. In (a) the vertical axis represents the SSC (Side scatter) and the horizontal axis represents the intensity of fluorescence linked to ALDH expression. Log scales were used. The proportions of cells expressing ALDH were a mean of 7 evaluations +/- SD calculated from human donors aged 45 to 63. In this group, approximately 1.6% of the cells were expressing ALDH as measured using the Aldefluor reagent. The percentages were varying from one sample to the other, according to the muscle of origin, and status of the donors

Characterization of cells by association of markers

Some populations of dissociated cells could be characterized by the association of markers, e.g. one or two extracellular markers and the expression of ALDH. As presented in Figure 2b, different markers could be associated to the expression of ALDH.

The co-expression of CD34 or its absence delineated two sub-populations (Figures 2 and 3). The relative proportion of total ALDH⁺ cells varied according to the anatomical origin of the tissue, while ALDH⁺/CD34⁻ cells represented 12% to 30% of the total ALDH⁺ cells (Figure 3A). Macaque skeletal ALDH⁺CD34⁻ cells predominantly exhibited SSC_{low} and FSC_{low} characteristics, while Macaque atrium ALDH⁺CD34⁻ cells constituted a subset with intermediate-to-high SSC and FSC characteristics. Macaque ventricular ALDH⁺CD34⁻ cells exhibited more heterogeneous SSC and FSC properties without forming a defined population (Figure 3C).

Some markers are regularly expressed on ALDH⁻ cells (such as the endothelial marker CD31), and some are consistently or preferentially expressed on ALDH⁺ cells (such as CD9, CD10, CD36, CD44, some CD49, CD140b, CD184, ...). We have noted that the association between markers is never complete, i.e. only a proportion of the ALDH⁺ cells expressed a given marker. As examples, in Humans, significant proportions of ALDH⁺ cells (i.e. more than 20%) were associated to the following markers (in ascending order) : CD36 < CD29 < CD9 < CD44 < CD10 < CD49e < CD34 < CD47. As another example, the majority of ALDH

cells (i.e. more than 50%) were associated with one of the following markers (in ascending order): CD44 < CD10 < CD49e < CD34 < CD47.

Some ALDH⁺ cells, that represent a minority among the whole ALDH⁺ cells (up to 20%), were also associated in Humans with at least one of the following markers (in ascending percentage of association): CD106 < CD56 < CD71 < CD31 < CD49a < CD140a < CD143 < CD309 < CD 49c < CD146 < CD61 < CD140b < CD184 < CD105 < CD49f < CD36.

This observation (Figure 3) does not disqualify some populations that look underrepresented, because the relative percentage of a cell population is not always representative of the final effect that it may produce.

Based on the functional, discriminative role of CD34 observed in populations extracted from human skeletal muscle, we identified populations associating some markers with the expression of ALDH and the expression of CD34 [ALDH⁺/CD34⁺] or its absence [ALDH⁺/CD34⁻] (Figure 2c and 4). As an example, Figure 2c displays the association between a given marker and the whole population of Human cells expressing ALDH and not expressing CD34 (i.e. [marker] x [ALDH⁺/CD34⁻]). A significant proportion of ALDH⁺/CD34⁻ cells (i.e. between 10 to 20%) were associated to the following markers (in ascending order) : CD140b, CD56, CD106, CD10, CD309, CD143, CD49a. Significant proportions of ALDH⁺/CD34⁻ cells (i.e. more than 20%) were associated to the following markers (in ascending order) : CD49e < CD71 < CD146 < CD49c < CD140a < CD105 < CD61 < CD31 < CD49f < CD44 < CD29 < CD36 < CD9 < CD184 < CD47. As another example, the majority of ALDH⁺ / CD34⁻ cells (i.e. more than 50%) were associated with one of the following markers (in ascending order): CD44 < CD29 < CD36 < CD9 < CD184 < CD47.

As presented in Figure 4, differences were noted between cell suspensions, depending on the anatomic origin, the animal species, and the combination of markers under scrutiny. Among tissues and species, the ALDH⁺/CD34⁺ populations are the more homogenous, since they are frequently associated to the expression of CD49e (integrin alpha 5) and CD140b (PDGF beta receptor), with or without CD10 (CALLA) and CD106 (VCAM-1). The ALDH⁺/CD34⁻ populations present common general features such as the expression of CD10 and CD44 in all tissues and species, but they are associated with specific features in skeletal muscle tissue such as expression of CD9 (MRP-1), CD56 (NCAM), CD184 (CXCR4) in Macaque, completed with CD31 (PECAM), CD36 (PASIV), CD49a (integrin alpha1), CD49c (integrin alpha3), CD49f (integrin alpha6), CD71 (Transferrin receptor), CD106, CD140a (PDGF alpha

receptor), et CD146 (MCAM) in Human. Therefore, ALDH⁺/CD34⁻ populations from cardiac and skeletal origin seem differentially defined, suggesting differential biological properties or functions. Differences in proportions of markers may be also noted between the cell populations extracted from skeletal muscles from Human and Macaque origins, but the most exclusive markers CD9 and CD184 were unchanged. Among markers, the combination of integrin isoforms within the whole family (CD49) helps differentiating cells from distinct origins. While expressed on 20-30% of cells extracted from ventricles and auricles, CD49a and CD49f are mainly expressed by ALDH⁺/CD34⁻ cells and CD49e by ALDH⁺/CD34⁺ cells extracted from skeletal muscles (Figures 2 and 4). Finally, in a given muscle tissue, some differences are observed between populations on the basis of CD34 co-expression. The ALDH⁺/CD34⁺ population is generally associated to the expression of CD49e and CD140b. The ALDH⁺/CD34⁻ population harbors common markers (CD10, CD44) and some that are more exclusively observed in skeletal muscle (CD9, CD56, CD184).

These data suggest that, thereafter, specific sub-populations of ALDH⁺ cells may be prepared by a person skilled in cell culture and/or cell selection, such as flow-cytometry-based sorting, or immunomagnetic selection using magnetic beads linked to specific antibodies or reagents (see example below). Of particular interest are the populations that associate ALDH expression, the lack of CD34 expression, and one or more of the following markers: CD106, CD56, CD309, CD143, CD49e, CD71, CD146, CD49c, CD140a, CD140b, CD49a, CD105, CD61, CD31, CD49f, CD44, CD29, CD36, CD9, CD10, CD184.

Sorting of populations using flow cytometry

Based on the combination of expression of ALDH and absence of expression of CD34, cell populations can be defined and sorted using flow cytometry. Figure 3, upper panels present the delineation of populations according to the associated fluorescence (CD34 in vertical axis, ALDH in horizontal axis). This delineation defines clusters of cells. These are projected to the physical definitions of the cells (side scatter, SSC and forward scatter, FSC) on bottom panels, showing homogenous populations of cells with low SSC and low FSC. As an example, the sorting of ALDH⁺/CD34⁺ and ALDH⁺/CD34⁻ cell populations extracted from Fascia Lata muscle biopsies of 7 patients, aged 47-63 years, provides one global population of ALDH⁺ cells representing 2.48±0.99% of the total Human mononucleated cells, out of which 2.02±0.9% are ALDH⁺/CD34⁺ and 0.46±0.12% are ALDH⁺/CD34⁻.

Similarly, a person skilled in cell selection would be able to prepare populations that associate to ALDH expression, and/or the lack of CD34 expression, and one or more of the following markers: CD106, CD56, CD309, CD143, CD49e, CD71, CD146, CD49c, CD140a, CD140b, CD49a, CD105, CD61, CD31, CD49f, CD44, CD29, CD36, CD9, CD10, CD184, CD56, CD106.

Preparation of cells using Ficoll separation

Alternatively, the preparation of the cells may first include a centrifugation onto a Ficoll layer (see materials and methods). The living mononucleated cells were harvested, washed and concentrated for any further purpose. As presented in Figure 4, the use of a Ficoll gradient allowed separating the fraction of living cells from a fraction that may contain debris, dying, apoptotic or dead cells. The use of Ficoll gradient fractionation allowed enrichment in cells expressing ALDH by a factor between 2 to 5. This methodology improved the purity and the richness of cell preparations and would make them more suitable for direct injection in the tissues either by direct or systemic ways.

Cardiogenic commitment of selected cell populations

The cells extracted from skeletal muscle biopsies, or given populations prepared using flow cytometry-based methodologies were treated using one or several cytokines for one to several days, as presented in Figure 6, before being analyzed for the proportion of cells expressing specific, i.e. cardiac markers using flow cytometry.

In Macaque cells, as presented in Figure 6B, 6D, 6E and 6F, the addition of recombinant BMP-2 modifies the proportion of cells expressing the cardiac marker GATA-4

As presented in Figure 6D, the addition of bFGF, BMP-2 and IGF-1 led to a 30 times increase in the proportion of cells expressing the cardiac marker NKx2.5 (Figure 6A, in presence of bFGF alone).

As presented in Figure 6F, the addition of BMP-4 modifies the proportion of cells expressing the cardiac marker Islet-1.

It should be noted that the effect of the cytokines on the cardiogenic differentiation of these cells, as observed through the expression of NKx2.5, is obtained upon incubation times varying from 12 to 168h. The efficient cytokines are chosen among the list comprising BMP-2, BMP-4, IGF-1, bFGF.

The percentages of cells expressing the cardiac markers GATA-4 and Nkx2.5 have been assessed by flow cytometry 48 and 72 h after the setting of cell culture and the supplementation with the cocktail of cytokines (bFGF, IGF-1, BMP-2, BMP-4). These percentages were evaluated using Human cells without the step of selection (Figure 7), and
5 Macaque cells without the step of selection (Figure 8). When the cocktail is renewed every day, there is a trend for obtaining higher percentages of cells expressing the cardiac markers than when the cocktail has been added only once at the onset of cell culture. Because cells are proliferating, their number increases between 48 and 72h, and, because the percentage of cells expressing cardiac markers is stable or increasing during this period of time, the absolute
10 number of cells expressing cardiac markers is more important. The percentages of human and mouse cells expressing cardiac markers have been also assessed after selection of ALDH⁺/CD34⁺ and ALDH⁺/CD34⁻ cells by flow cytometry (Figure 9). 48 h after setting of the culture and addition of the cocktail of cytokines (daily renewal), the percentages of cells expressing cardiac markers is high. While no consistent difference can be observed between
15 the initial populations, it should be noted that the final percentages of positive cells are much higher than the ones observed starting from non-selected populations of cells (Figures 7 and 8). The selection of cells on the basis of ALDH activity increases the yield of cells expressing cardiac markers.

In mouse cells, the effect of cytokine treatment was observed in vitro using the
20 cytofluorescence methodology. As presented in Figure 10, a mouse ALDH⁺/CD34⁻ cell population (prepared by FACS as provided above), when exposed to cytokines as above, expressed markers of cardiogenic differentiation, such as connexin-43 and alpha-actinin.

Preparation of committed cells

25 Following induction in culture, the cells prepared from skeletal muscle biopsies were harvested. This step can be achieved 12 to 168h after induction, using either enzymatic or mechanical detachment of the cells. The cells were gathered and concentrated in a medium that warranted their survival up to their use in vitro or in vivo. This medium was based on a synthetic formulation containing, minimally, salts, eventually complemented with amino-
30 acids and glucose. Typically, but not restrictively, a composition may contain MEM, DMEM, PBS, MCDB synthetic medium. The medium was completed with 0.5% serum albumin.

Following induction, the committed cell populations may be further enriched by selection, based on the expression of extracellular markers such as CD140a, CD106, CD172a. The

selection can be achieved using immunomagnetic beads or fluorescence associated flow cytometry

Implantation of committed cells in vivo

- 5 Table 6 recapitulates the several conditions tested for implantation of the cells, and the results in terms of engraftment and presence of cells harboring cardiac markers upon sacrifice of the animals.

Table 6

Cell Phenotype	Nb mice	Expansion in vitro (duration)	Cardiac Induction in vitro	Presence of grafted cells in vivo	Cardiomyogenic differentiation of grafted cells in vivo	Buffer
ALDH ⁺ (Fresh)	1	No	No	-	-	Aldefluor buffer
ALDH ⁺ (Frozen)	5	No	No	+	-	Aldefluor buffer
ALDH ⁺ /34 ⁻	2	48 hours	Yes	+	+	Aldefluor buffer
ALDH ⁺ /34 ⁺	1	48 hours	Yes	+++	-	Verapamil in PBS + 2% FBS
ALDH ⁺ /34 ⁻	3	48 hours	No	+++	-	Verapamil in PBS + 2% FBS
ALDH ⁺ /34 ⁻	2	48 hours	Yes	+++	+++	Verapamil in PBS + 2% FBS
ALDH ⁺	2	72 hours	Yes	+++	+++	Verapamil in PBS + 2% FBS

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The said cells were injected within the myocardial tissue of recipient animals. In the following but non limiting examples, cells have been prepared from non human primate biopsies, selected, committed in vitro using the cocktail of cytokines (see above), and injected into the heart tissue of immunodeficient mice.

- 15 Indeed, in a xenogenic context (donor and recipient belong to two different animal species), the recipient has to be immunodeficient to accommodate the transplantation without immune rejection. Cell transplantation can be also performed in an allogenic context, where donor and recipient belong to the same species but are two distinct and non-monozygotic individuals. Cell transplantation can be also performed in an autologous context, where donor and

recipient are the same individual. Cell transplantation can be also achieved within the myocardium of recipients affected by cardiac diseases, whether genetic, idiopathic, iatrogenic. Twenty-one days (Figures 11 and 12) and 30 days (Figure 13) after transplantation, animals were sacrificed, their hearts were processed and the presence of cells harboring specifically non-human primates proteins was demonstrated within the murine tissue using immunohistological procedures.

The injected cells did implant as concentrated clusters, or in a more dispersed manner within the grafted areas. A few days after injection, within delays from 2 days to several months, the injected cells expressed markers typical of structure (troponin, actinin) or function (connexin 43) of cardiomyocytes. The cells also expressed markers that are species-specific (lamin A/C in Primates) and therefore demonstrate their origin.

Figure 11 illustrates serial sections, allowing the identification of several markers in the same heart area. Non-human primate cells expressed a specific lamin A/C that stained their nuclei in green (left column), at distinct and discrete areas in the myocardium that corresponded to injection sites. The cardiac protein alpha-actinin was expressed in the cytoplasm of both murine (recipient) cardiac cells, and non-human primate (donor) cells. A majority of cells in the same area (right column) also expressed the cardiac marker Cardiac troponin I, and only a very small number of cells expressed a fast isoform of skeletal myosin heavy chain (a marker of differentiated skeletal muscle cells). These Figures illustrate the implantation of the said injected cells within clusters in recipient hearts, and the expression of cardiac markers, that suggest the commitment of the injected cells into cardiomyocyte-like cells *in vivo*.

Figure 12 (21 days after injection) and Figure 13 (30 days after injection) illustrate the implantation of said committed cells at different sites within mouse myocardium *in vivo*. Upon implantation, the donor cells specifically expressed a non-human primate lamin A/C protein that stained their nuclear membrane. The cells expressed a cardiac marker in their cytoplasm, the alpha-actinin, as did the murine cardiomyocytes in the surrounding (recipient) environment. Murine and non-human primate cells also expressed a cardiac membrane marker, connexin-43, that normally warrants electrical coupling between cardiac cells. Some of the cells expressing the non-human primate lamin A/C exhibited striations, which characterized the presence of sarcomeres and of a contractile apparatus. Some of these cells

were undistinguishable from the resident murine cardiomyocytes that constituted the majority of the murine heart tissue.

We compared the yield of ALDH⁺ cells obtained upon incubation of the Aldefluor substrate in presence of the classical, commercial Aldefluor buffer, and a home-made buffer containing PBS, 2% FBS, and verapamil (Figures 14-16). The verapamil concentration was 100μM (Figures 14 and 16) or was variable along a range (1-1000μM, Figure 15). In presence of the inhibitor DEAB, no shift of fluorescence was observed and almost no ALDH⁺ population was defined. In absence of DEAB, the use of verapamil buffer instead of the use of the classical Aldefluor buffer increased the number of cells expressing ALDH, whether originating from Macaque skeletal muscle (top panel) or Human cardiac muscle (bottom panel) (Figure 14). The assessment of different concentrations of verapamil (Figure 15, bottom panel) indicated that the highest percentages of Macaque skeletal muscle cells expressing ALDH were obtained using 10 to 250μM of verapamil. These concentrations doubled the percentages of ALDH⁺ cells, as compared to the yield obtained using the classical Aldefluor buffer (top panel). The use of verapamil buffer to identify the ALDH⁺ cells still allows their labeling and selection using other markers such as CD34 (Figure 16). Indeed, within skeletal muscle cells of Macaques, ALDH⁺/CD34⁺ and ALDH⁺/CD34⁻ populations can be clearly discriminated.

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CLAIMS

1. A skeletal muscle-derived cell population having cardiogenic capacities.
5
2. The cell population according to claim 1, wherein the cell population is an ALDH⁺, in particular an ALDH⁺/CD34⁻ skeletal muscle-derived cell population.
3. The cell population according to claim 1 or 2, said cell population being further
10 characterized by at least one of the following markers: CD49e, CD71, CD146, CD49c, CD140a, CD105, CD61, CD31, CD49f, CD44, CD29, CD36, CD10, CD106, CD140b, CD49a, CD143, CD309, CD56, CD9 and CD184.
4. A method for the production of a cardiogenic cell population, comprising providing a
15 skeletal muscle cell population, and culturing said skeletal muscle-derived cell population in vitro, thereby obtaining a cell population having cardiomyogenic differentiation abilities.
5. The method according to claim 4, wherein the skeletal muscle cell population is an
20 ALDH⁺/CD34⁻ cell population.
6. The method according to claim 4 or 5, wherein the cell population is sorted based on ALDH expression in a medium containing a calcium channel blocker, in particular Verapamil.
- 25 7. The method according to any one of claims 4 to 6, wherein the skeletal muscle-derived cell population is cultured in a cell culture medium comprising one or more differentiation and/or growth factors.
8. The method according to claim 6, wherein the differentiation and/or growth factors are
30 selected in the group consisting of BMP-2, BMP-4, IGF-1 and bFGF.
9. The method according to any one of claims 4 to 8, wherein the cell population is cultured *in vitro* at least 24 hours, in particular between 24 hours and 20 days.

10. A cardiogenic cell population derived from a skeletal muscle cell population, wherein said cardiogenic cell population is obtainable according to the method of any one of claims 4 to 9.

5 11. A cardiogenic cell population according to any one of claims 1 to 3 and 10, for use in the treatment of a cardiac defect, disease or pathology.

12. A composition comprising a cardiogenic cell population according to any one of claims 1 to 3 and 10, in a pharmaceutically or physiologically acceptable carrier, excipient or diluent.

10

13. A composition comprising BMP-2, BMP-4, IGF-1, and optionally bFGF.

14. The composition according to claim 12 or 13, comprising a muscle cell culture medium comprising with BMP-2, BMP-4, IGF-1, and optionally bFGF

15

15. A composition suitable for selecting an ALDH⁺ cell, in particular an ALDH⁺ skeletal muscle cell, comprising a calcium channel blocker such as Verapamil.

16. The composition according to claim 15, comprising Verapamil at a concentration of 0.5 to
20 500 μ M, in particular of 1 μ M to 250 μ M, in particular of 50 to 100 μ M, more particularly of 100 μ M.

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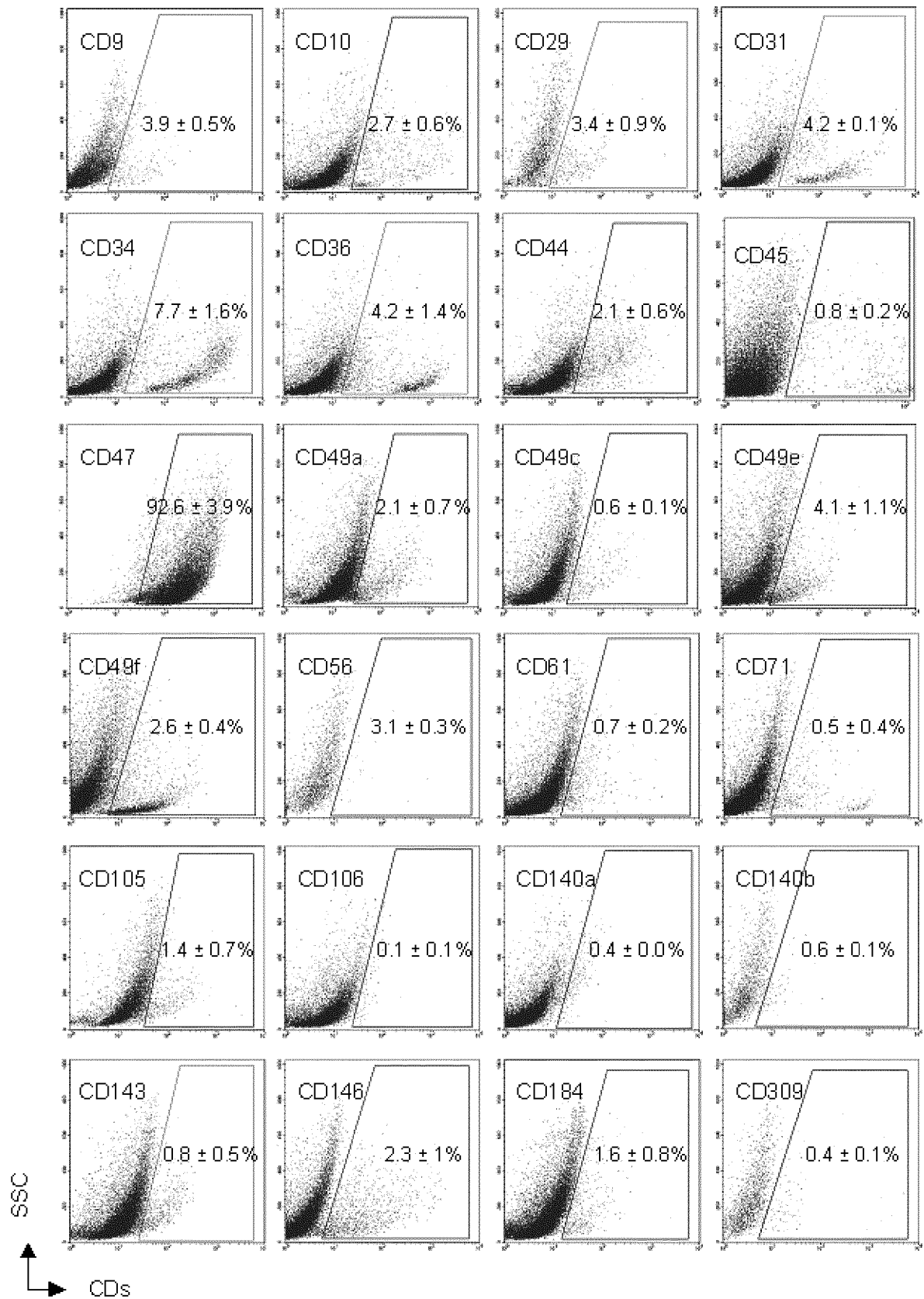


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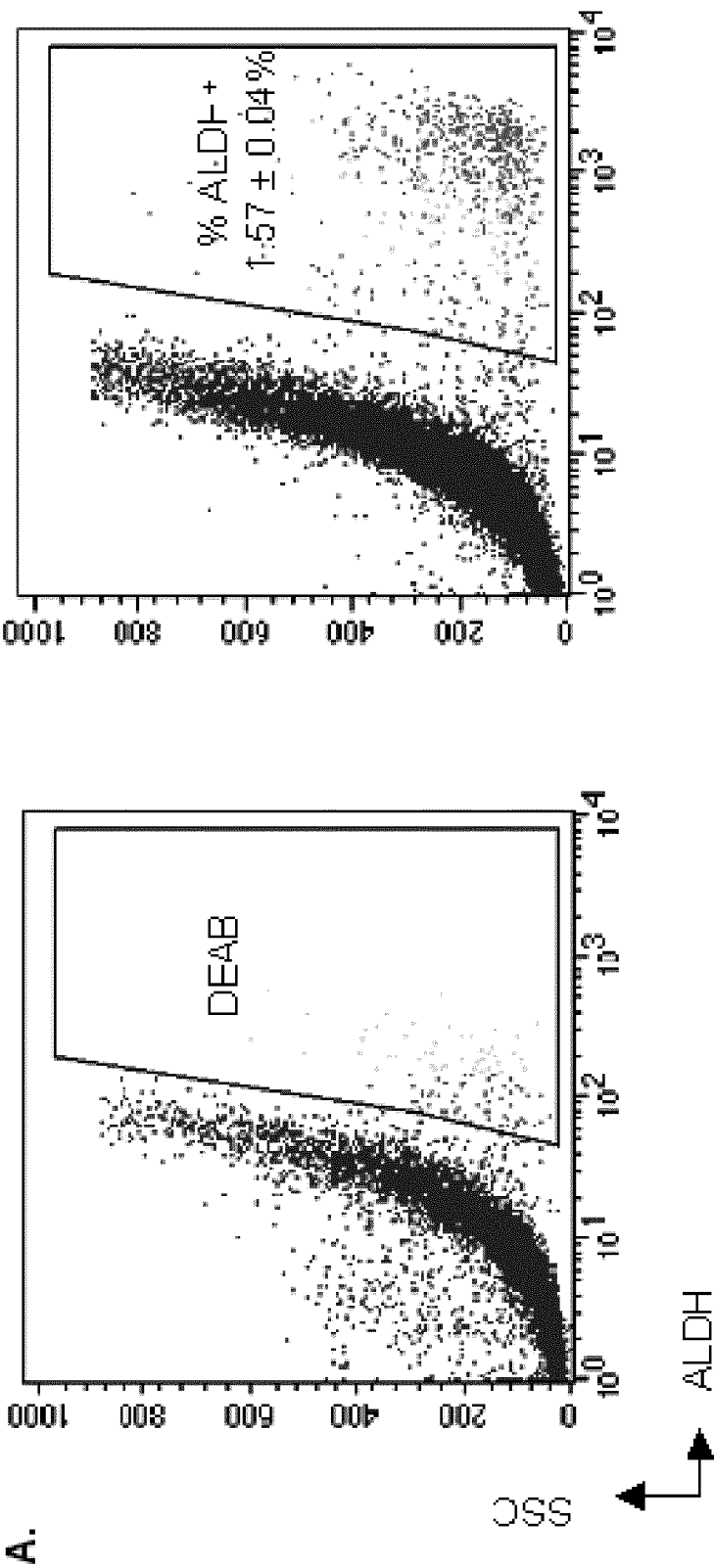


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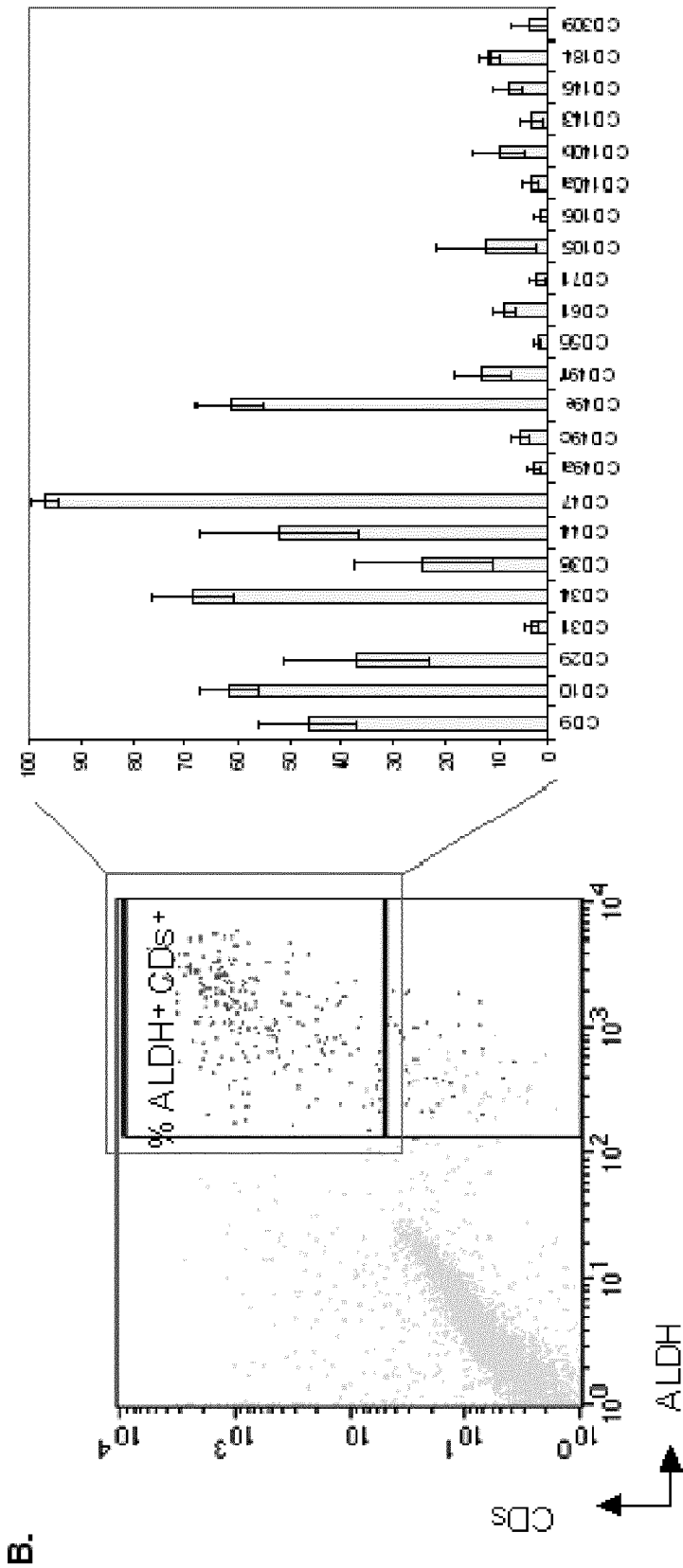


Figure 2 (cont.)

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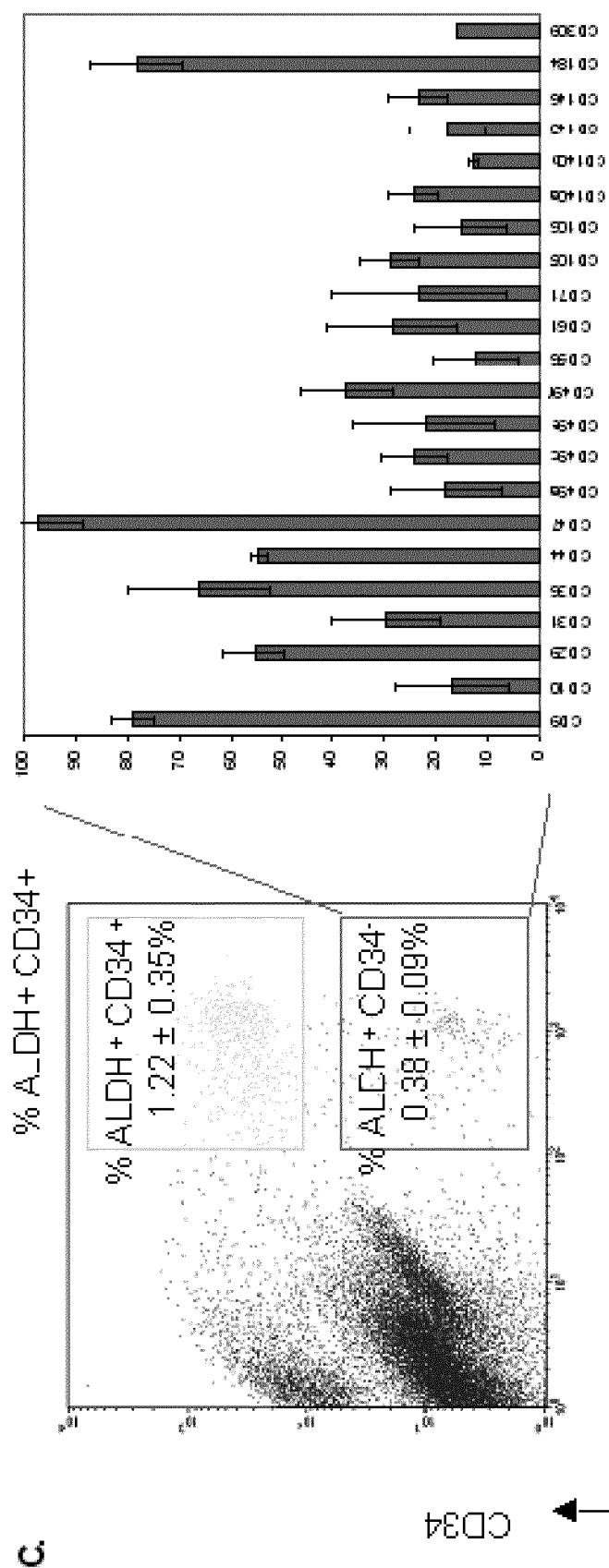


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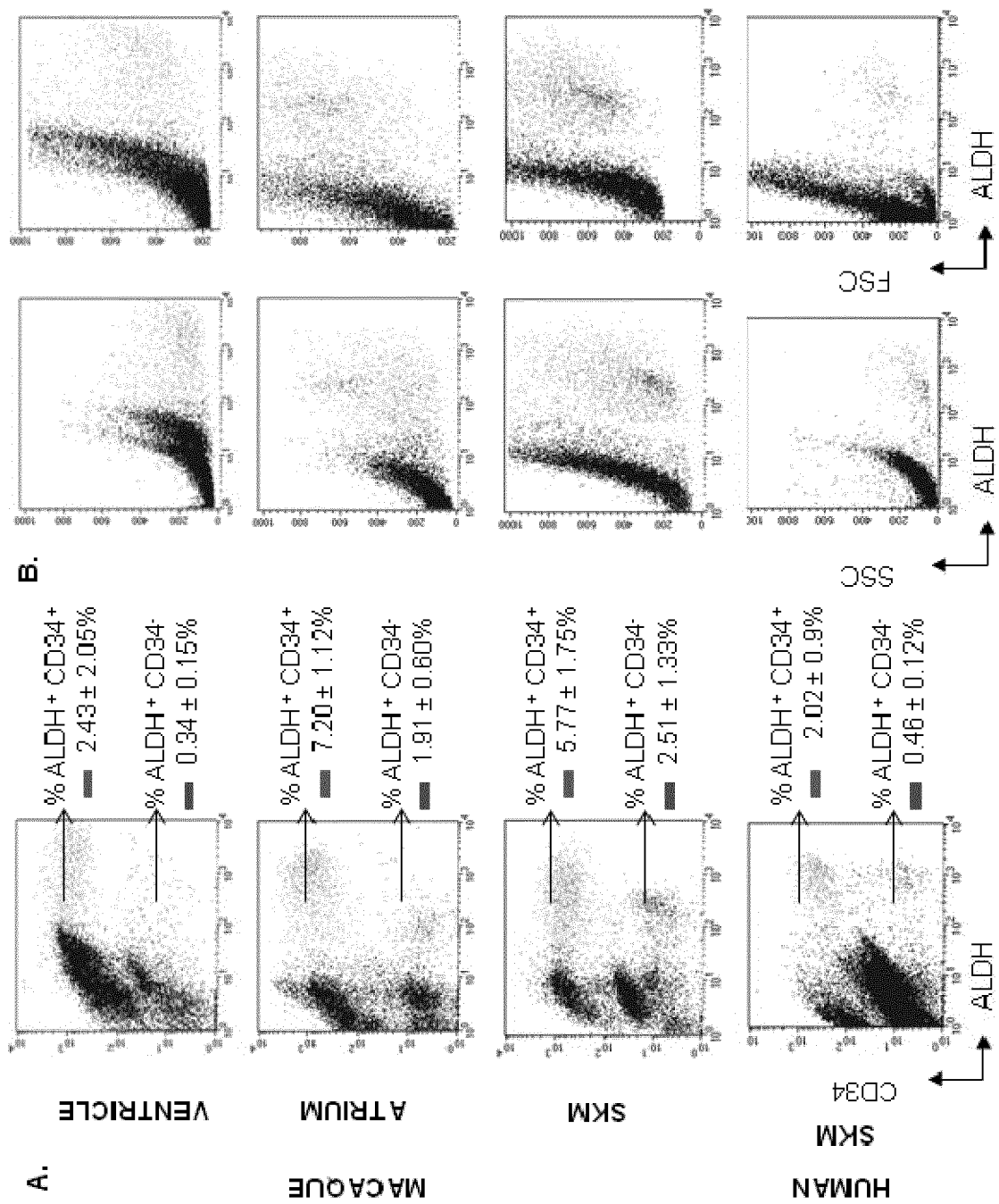


Figure 3

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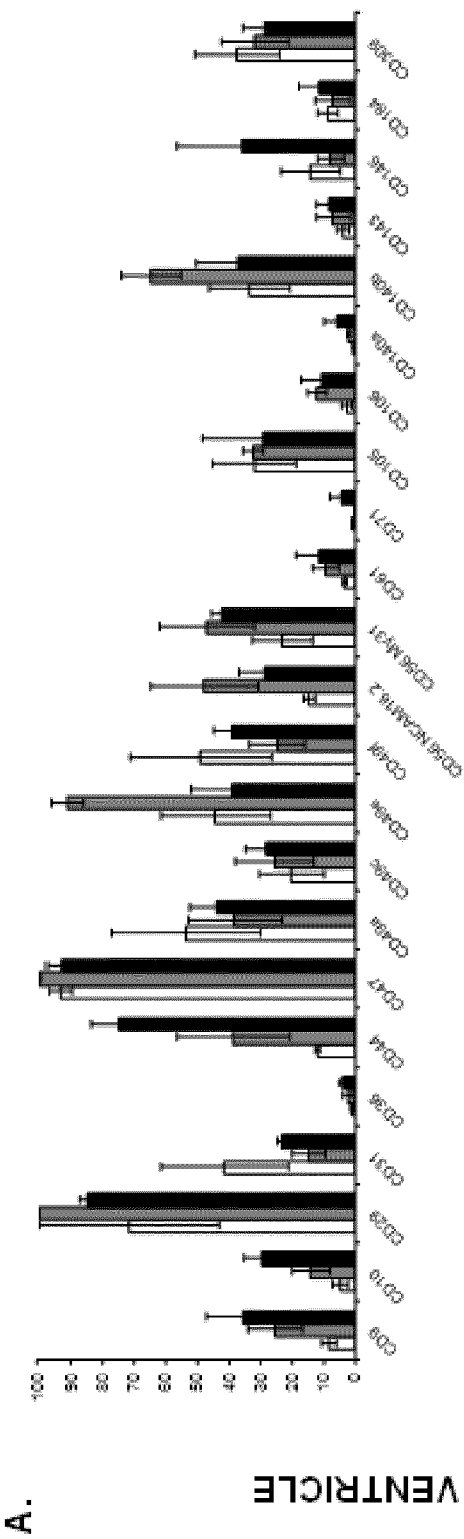


Figure 4

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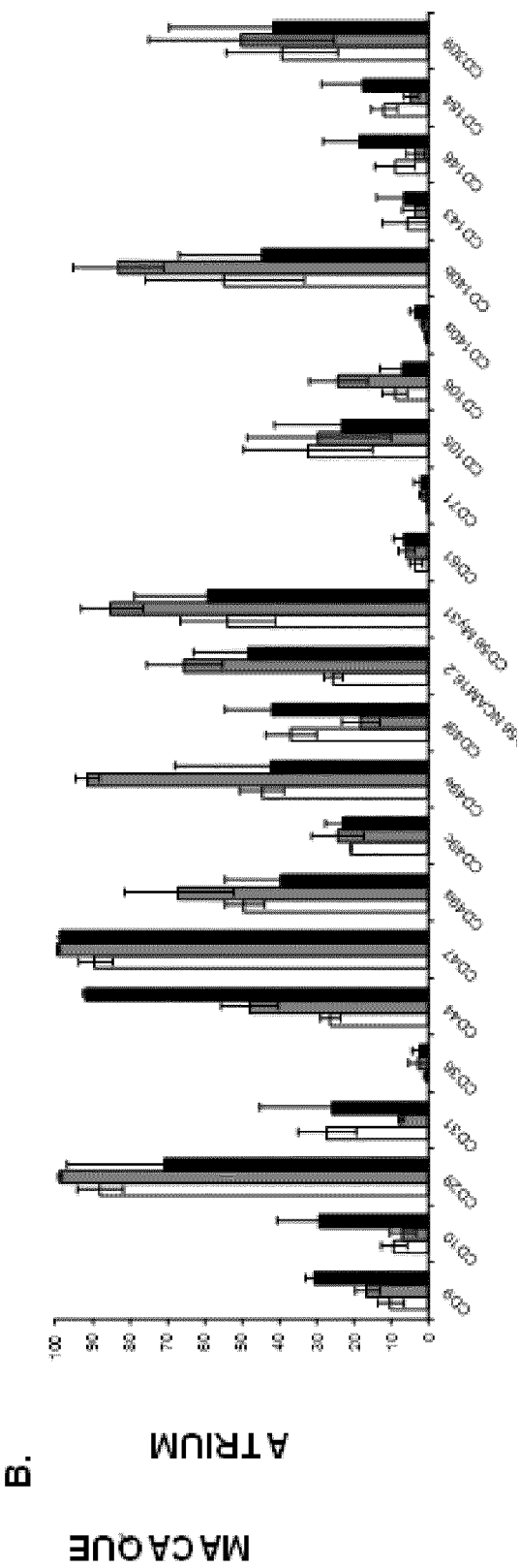


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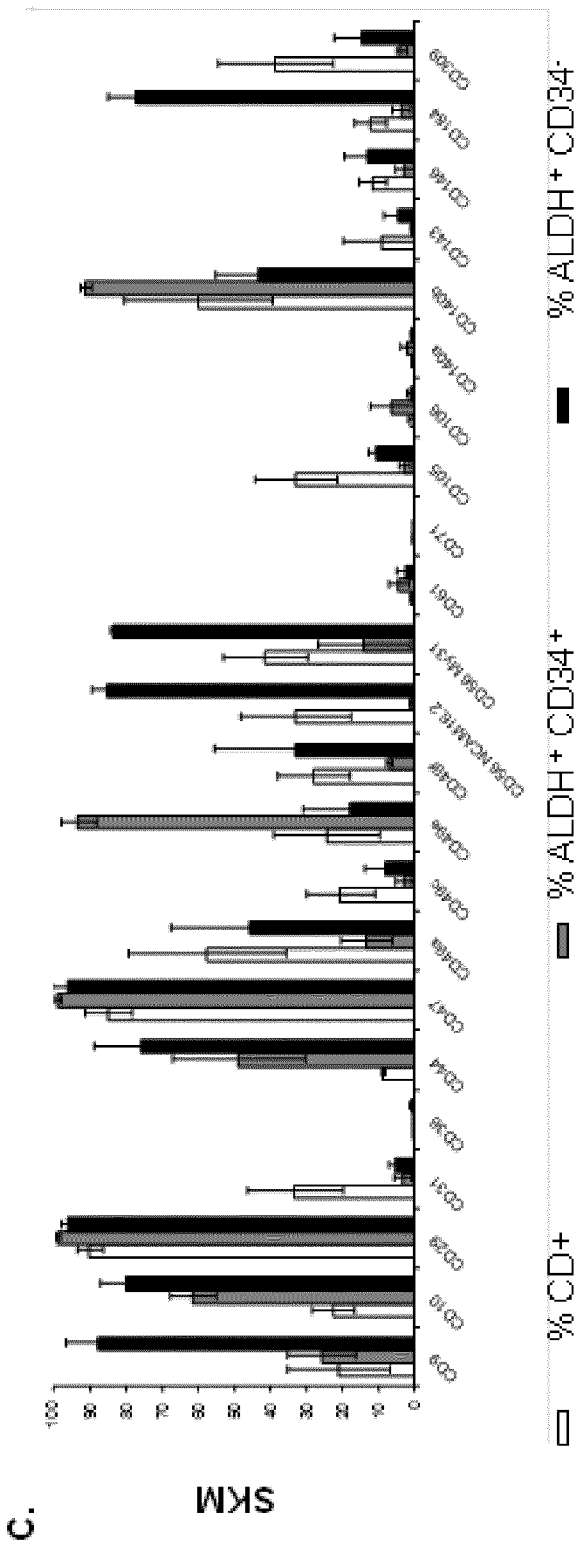


Figure 4 (cont.)

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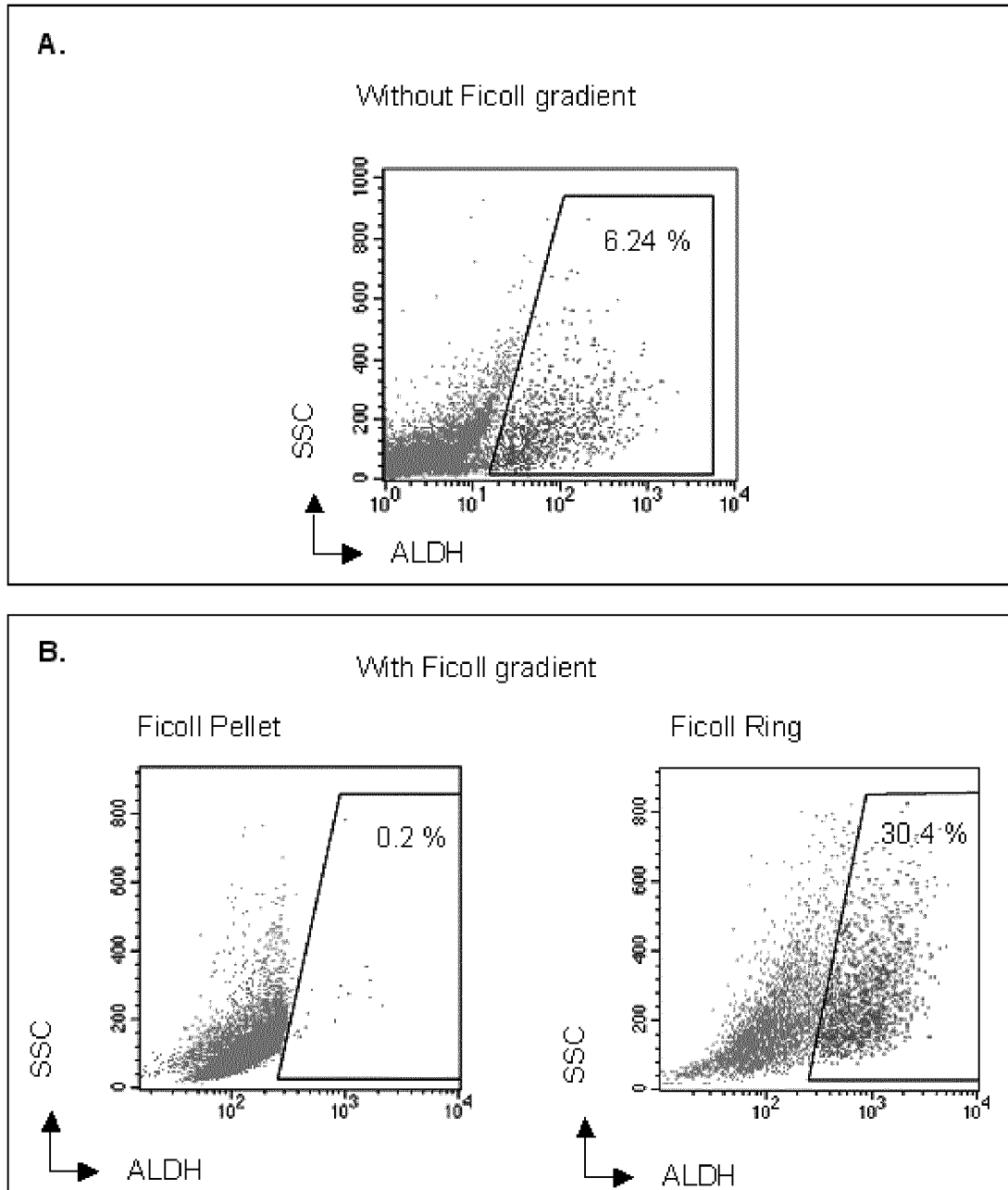


Figure 5

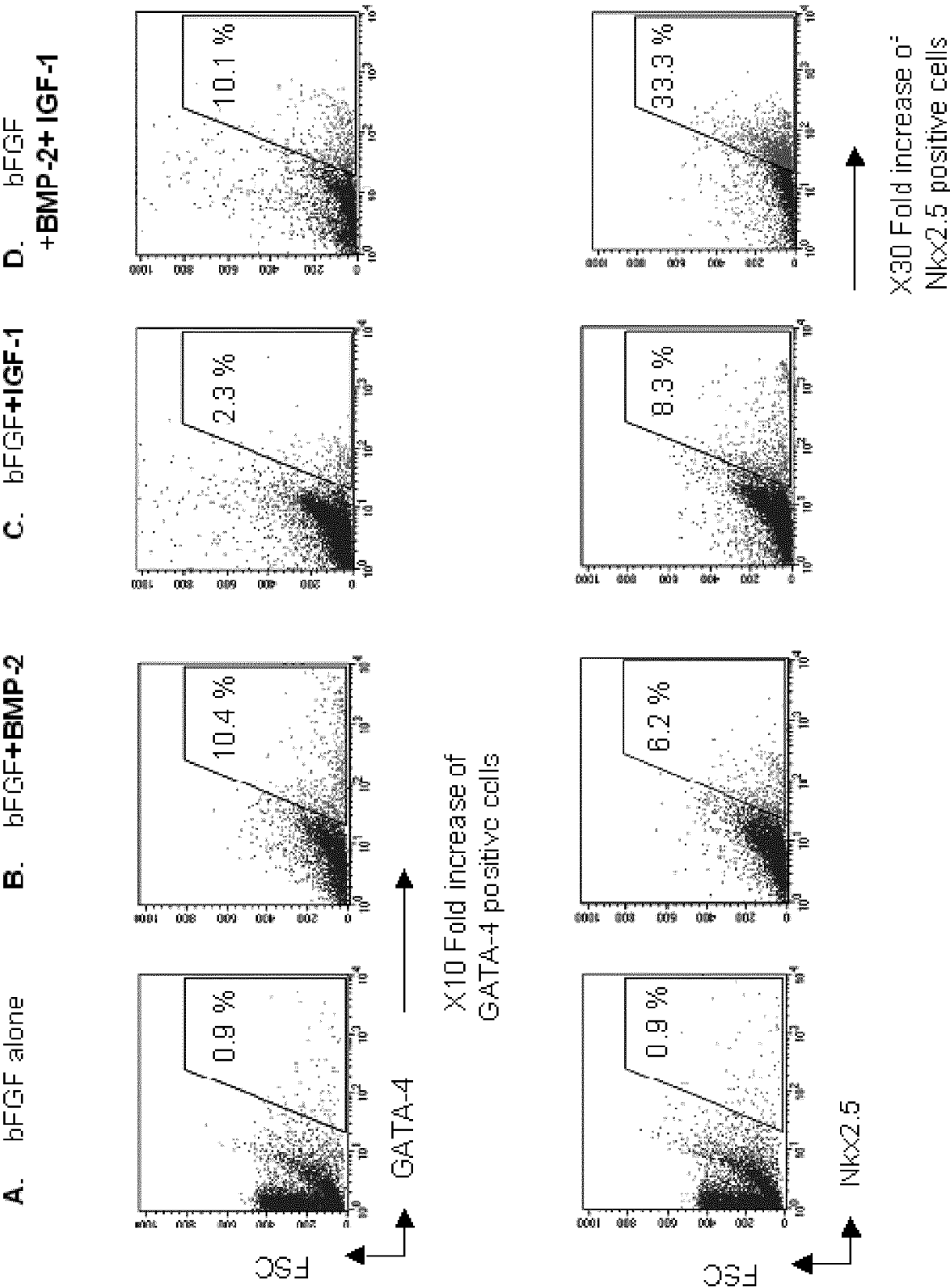


Figure 6

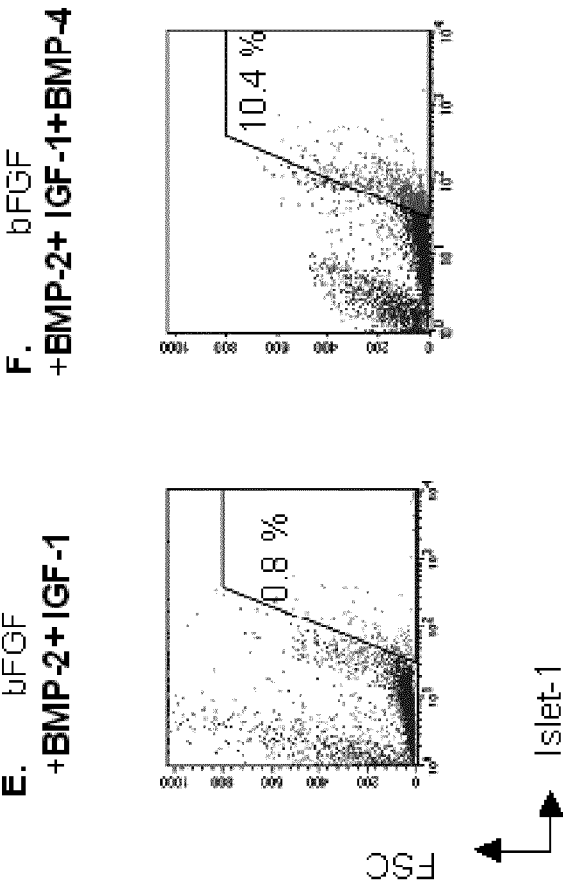


Figure 6 (cont.)

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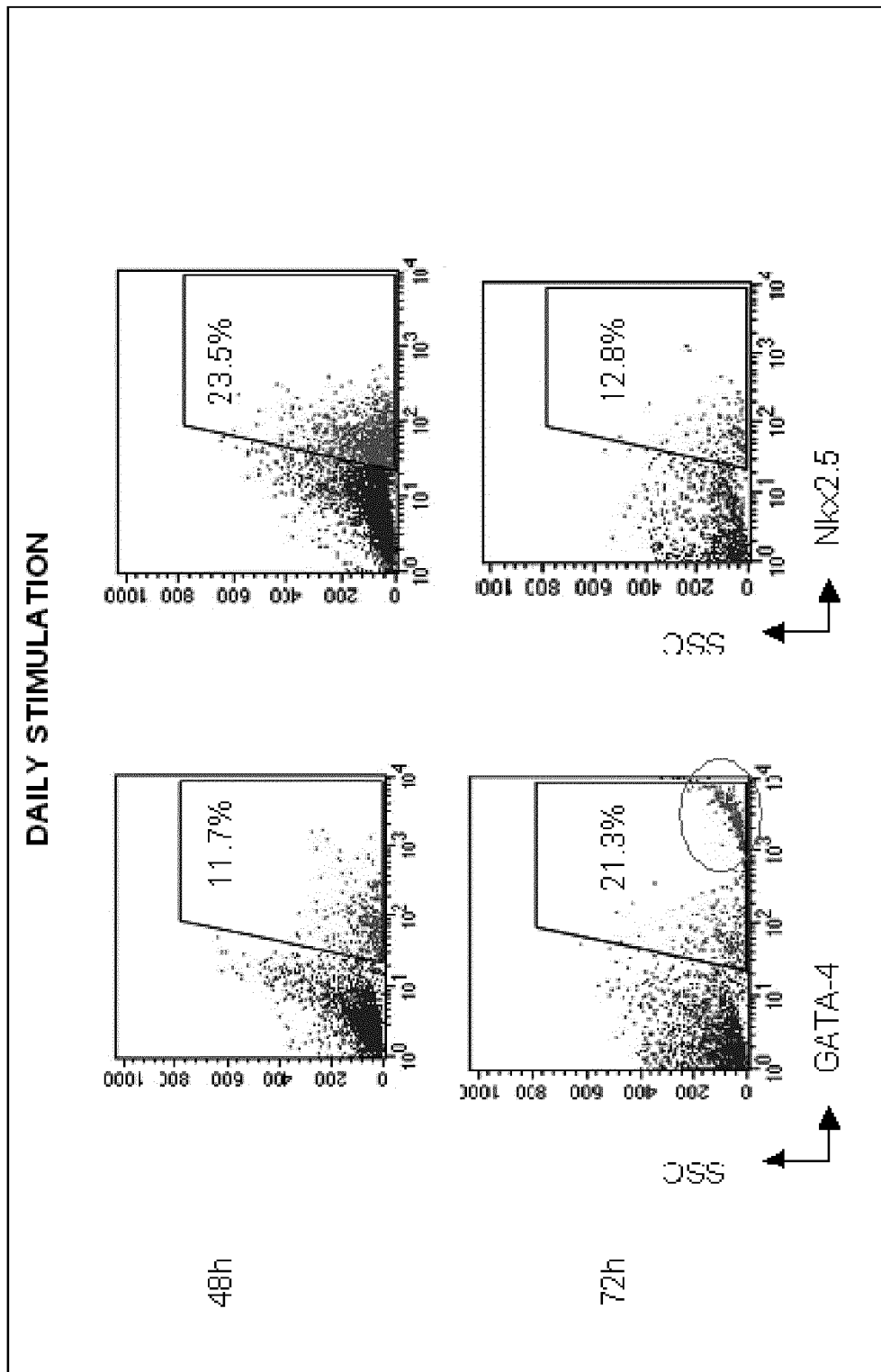


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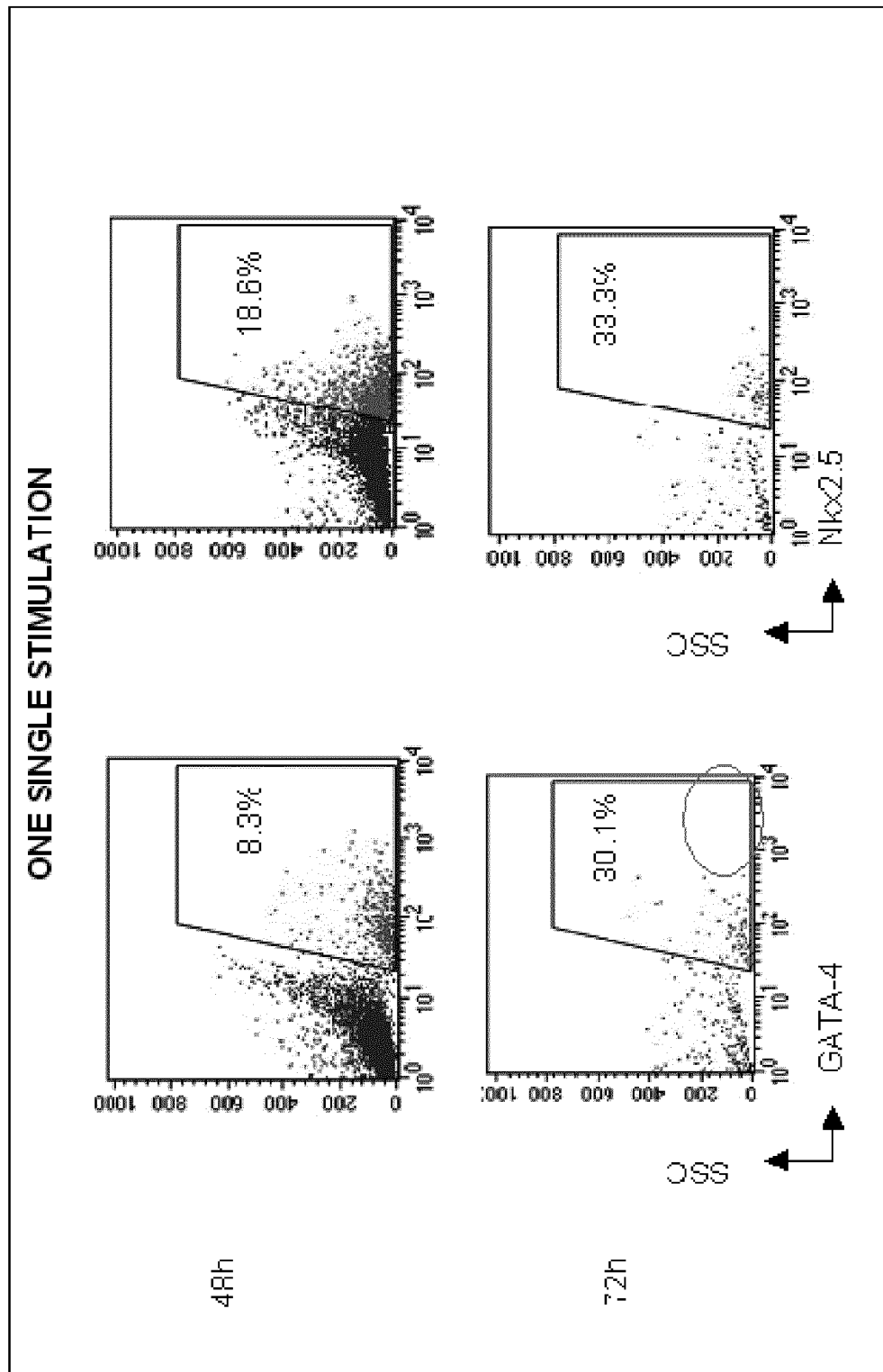


Figure 7 (cont.)

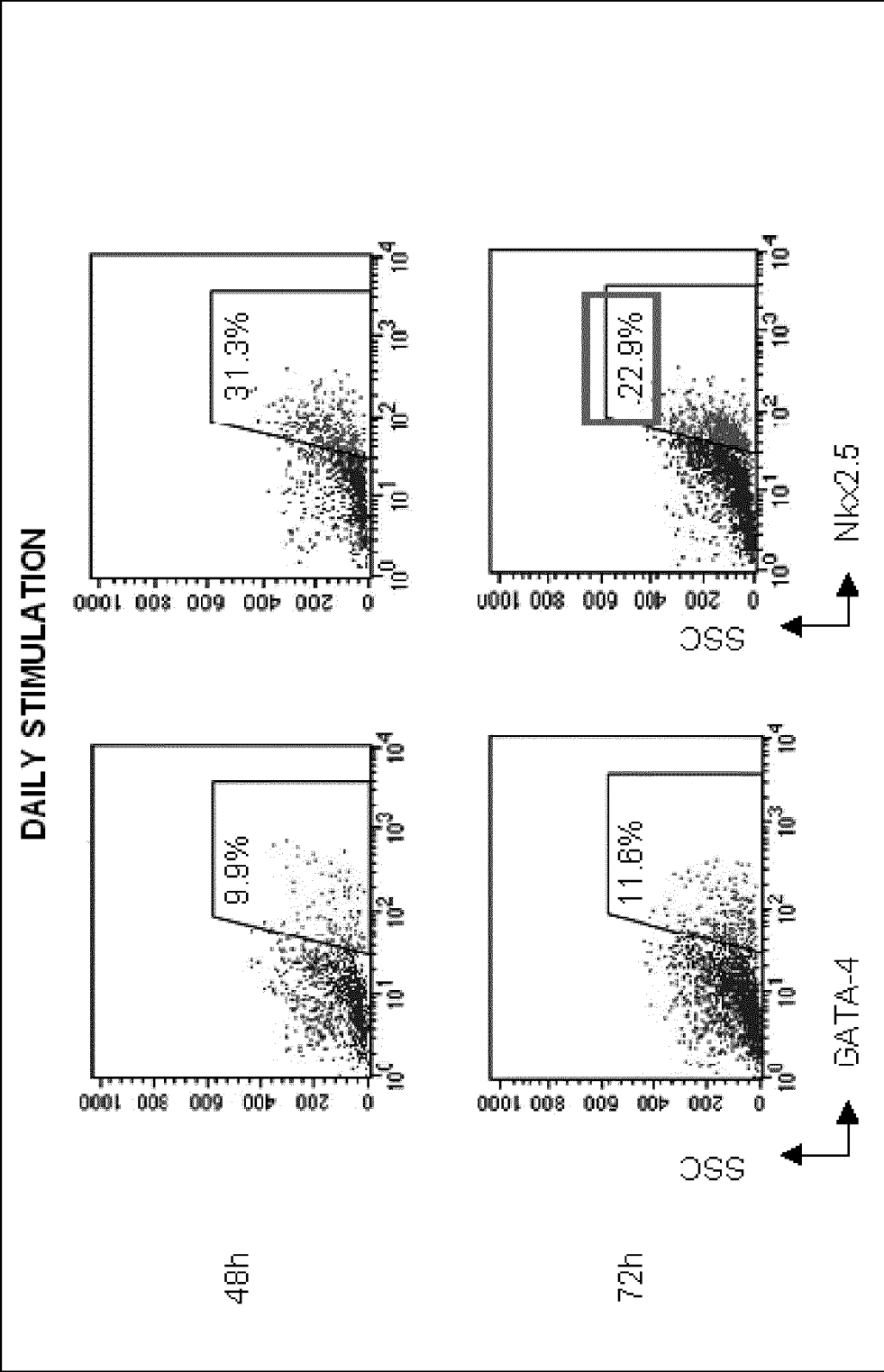


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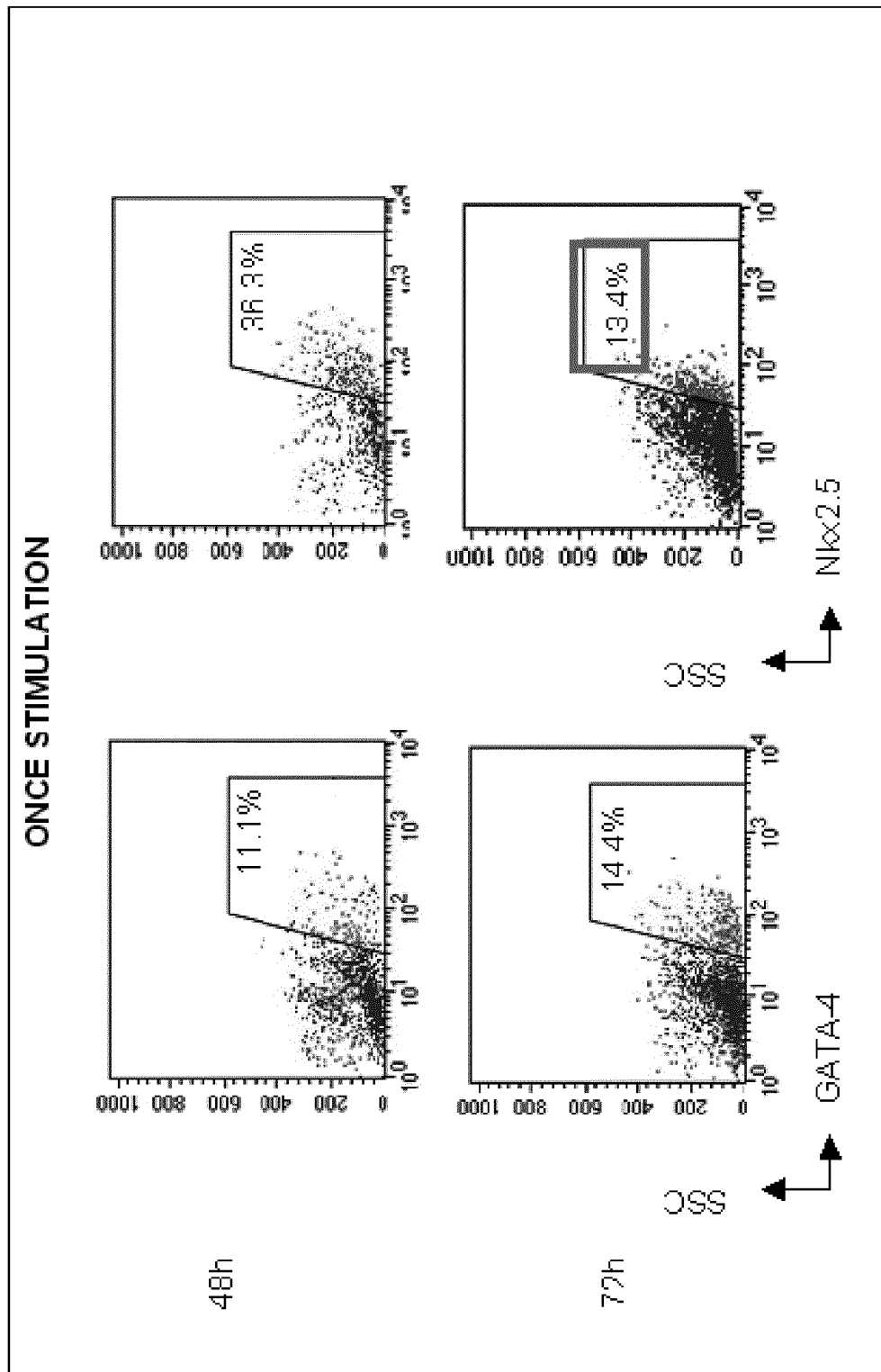


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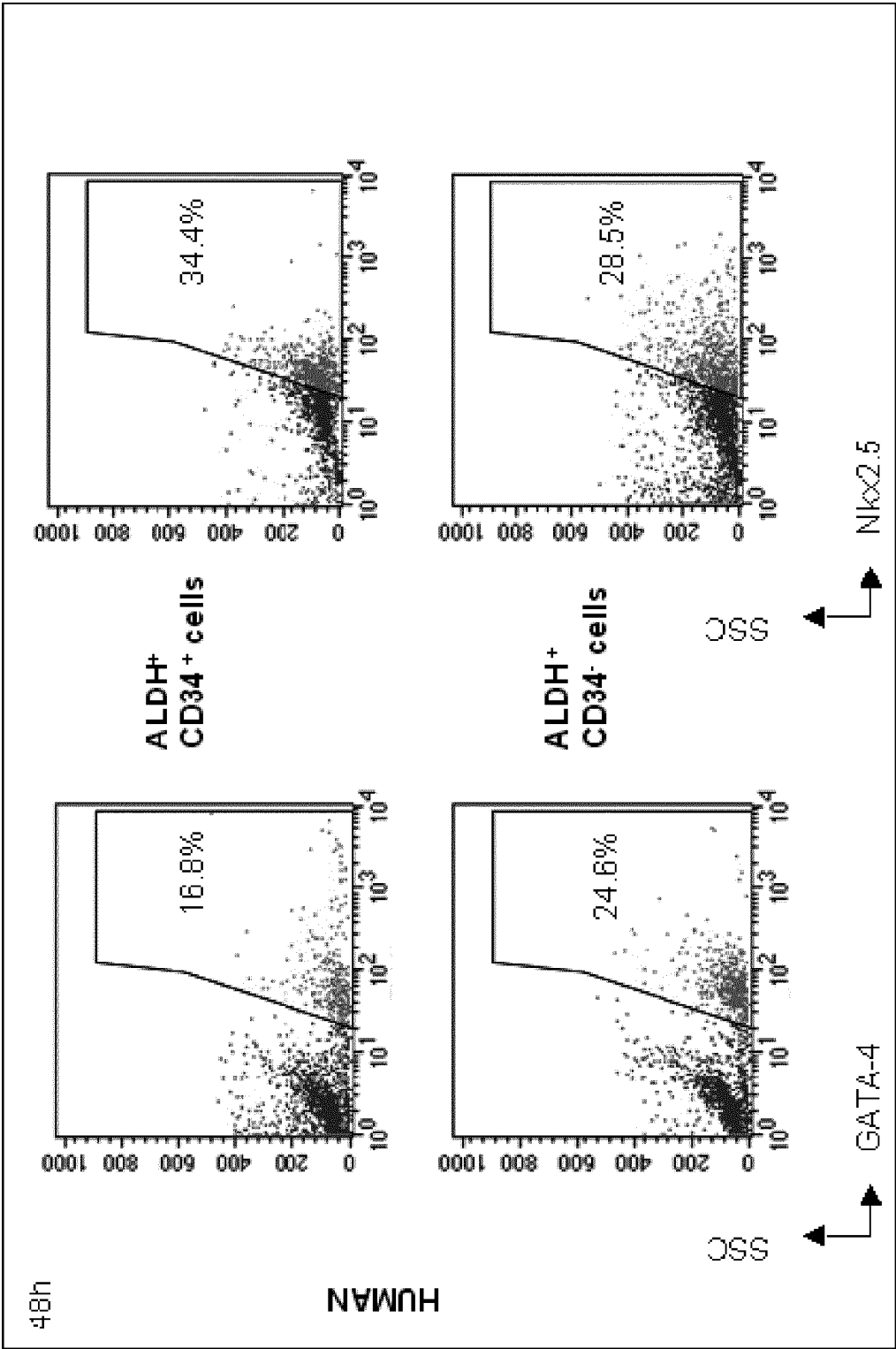


Figure 9

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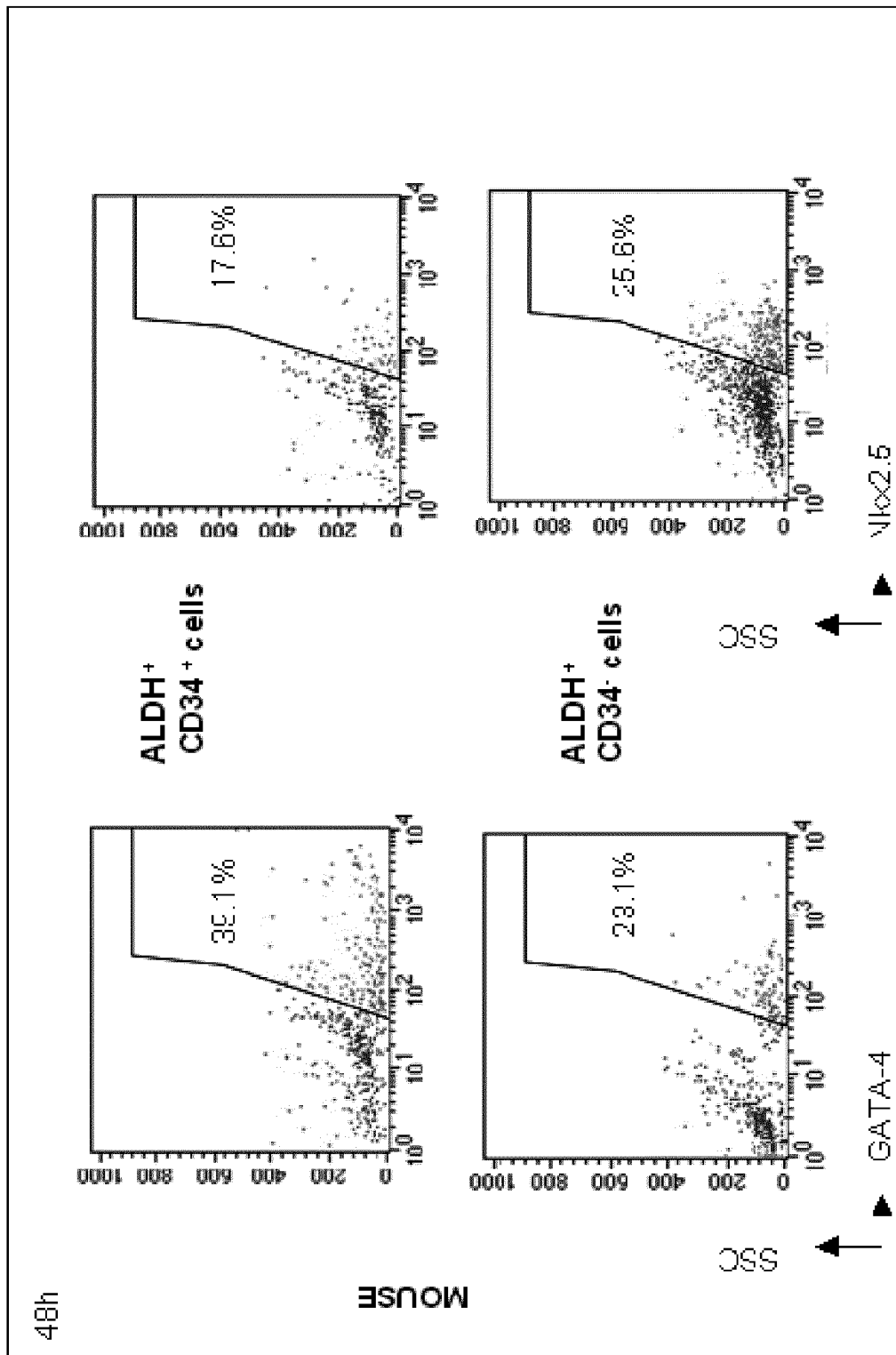
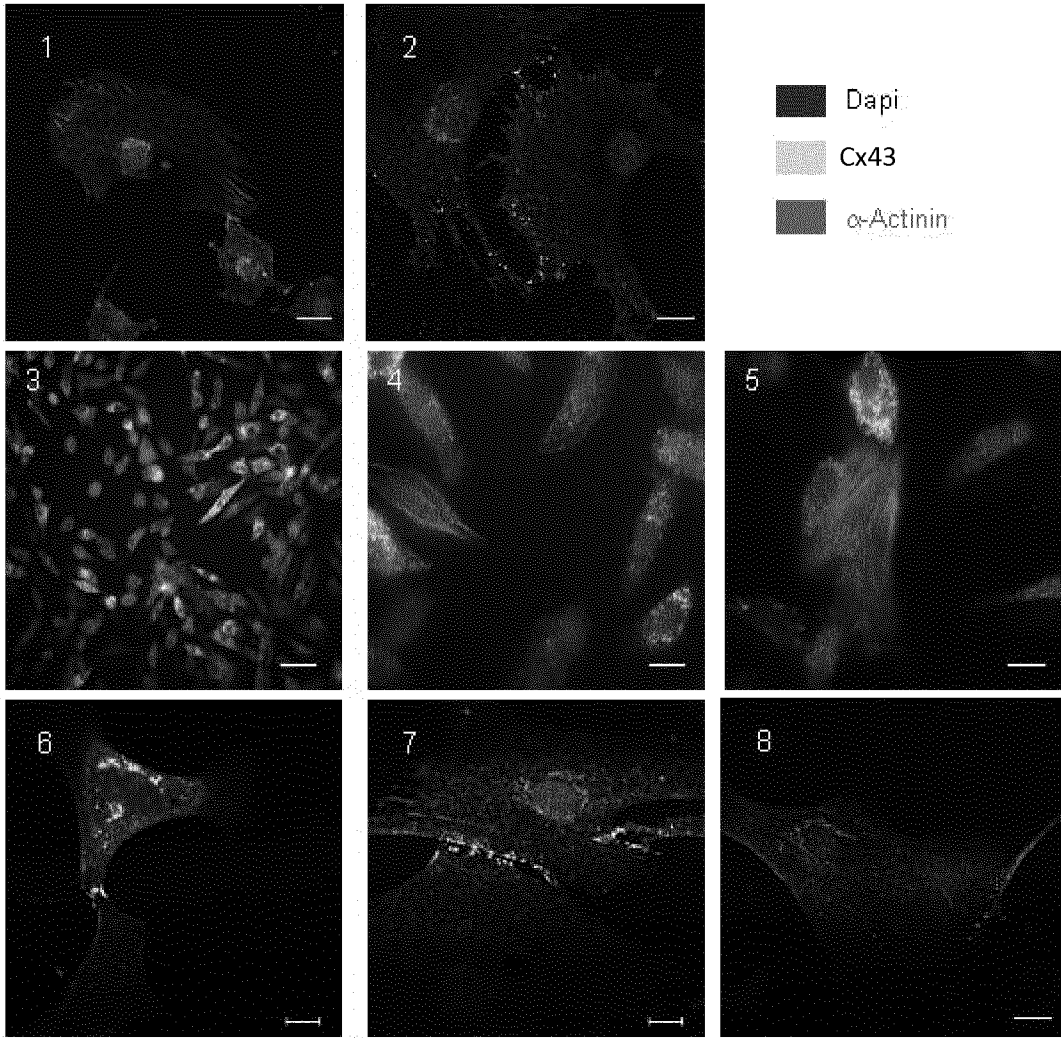


Figure 9 (cont.)

A.



B.

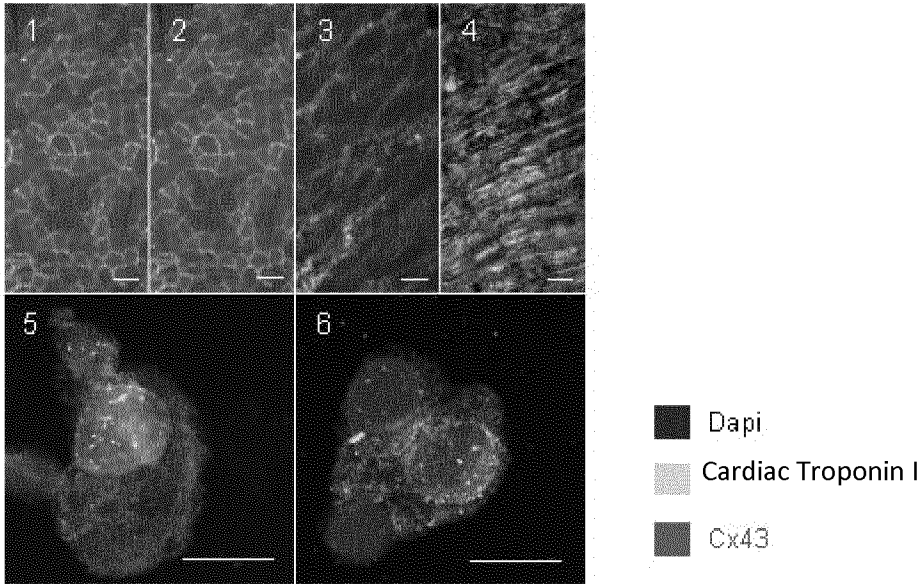


Figure 10

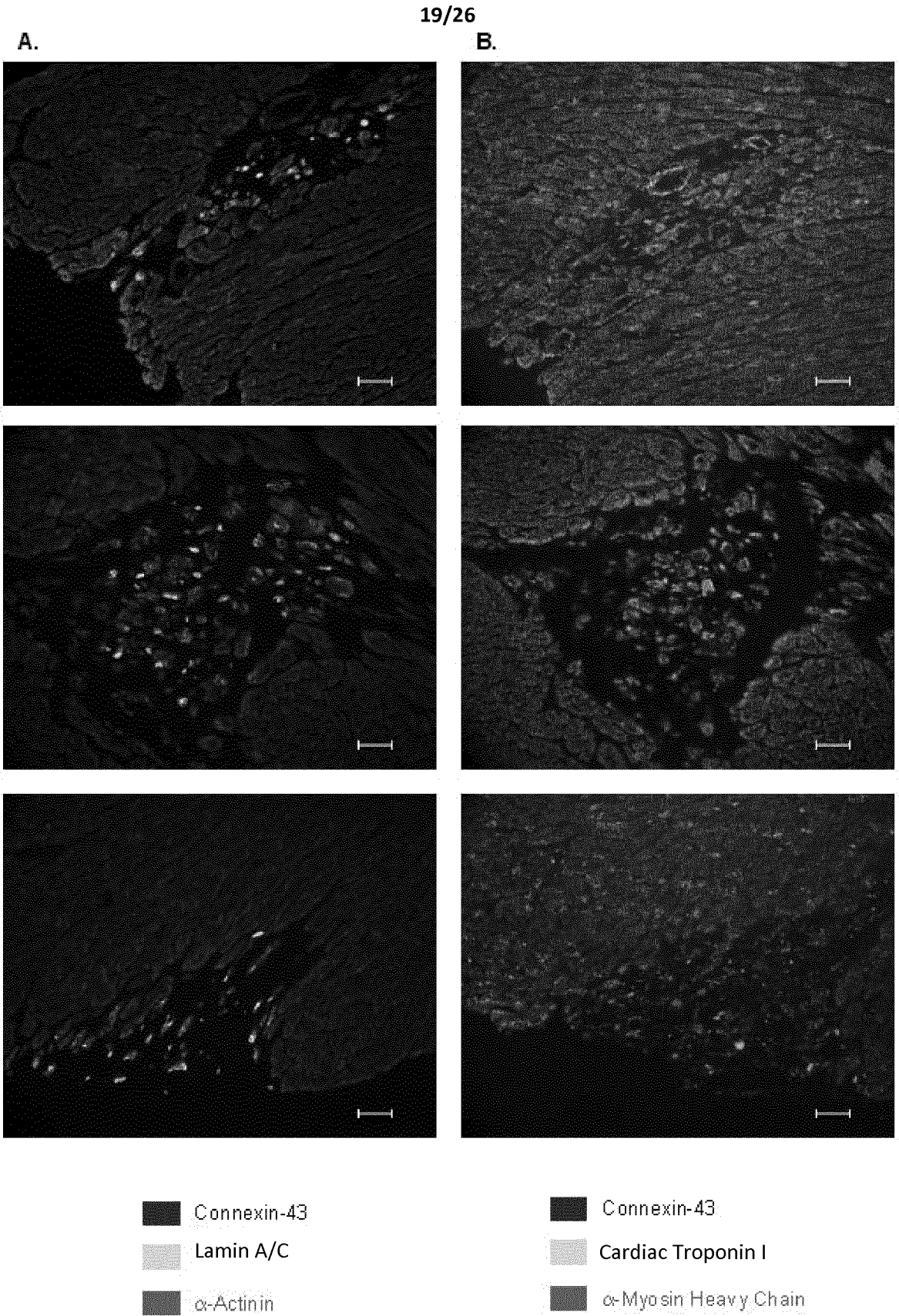


Figure 11

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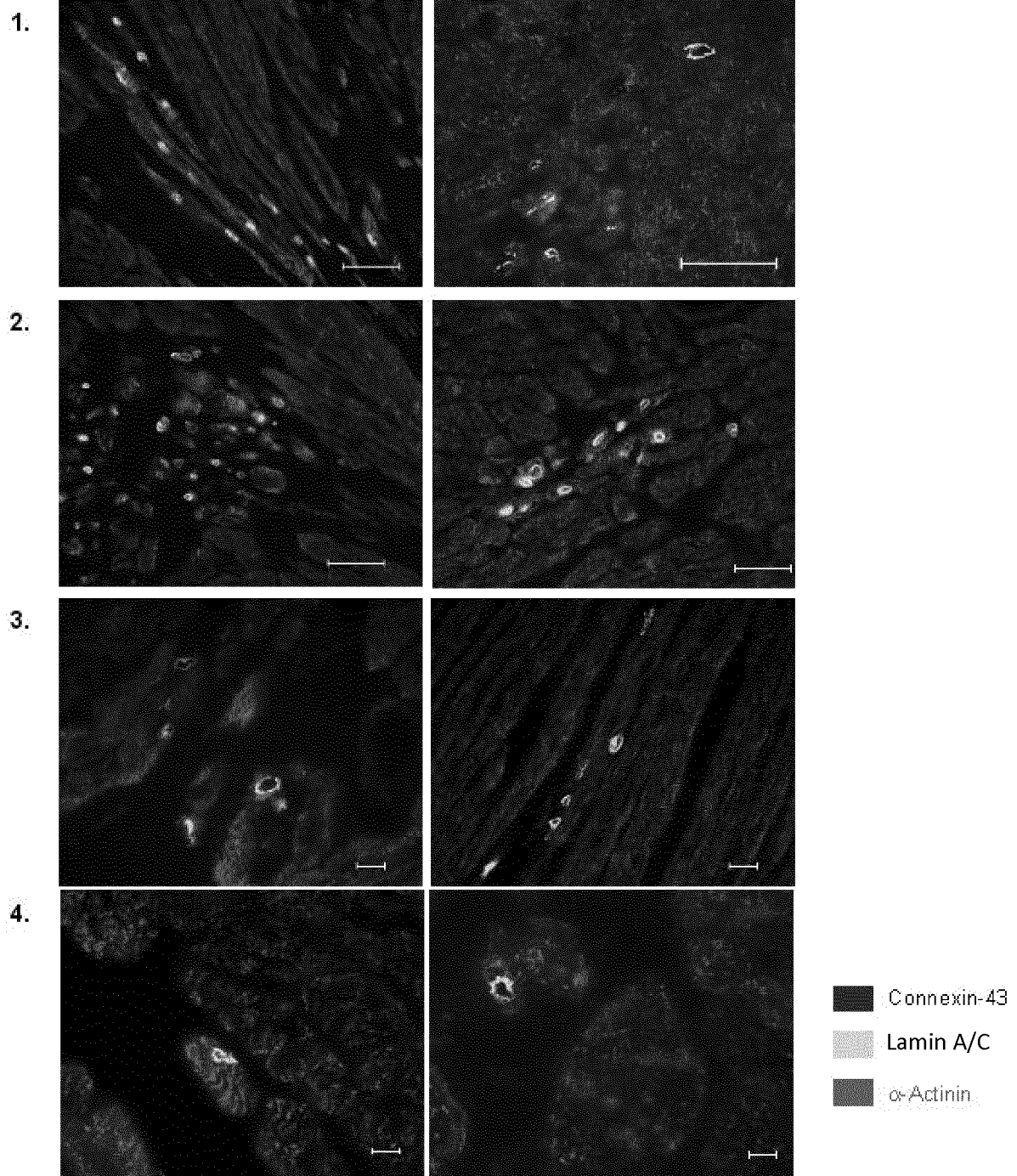


Figure 12

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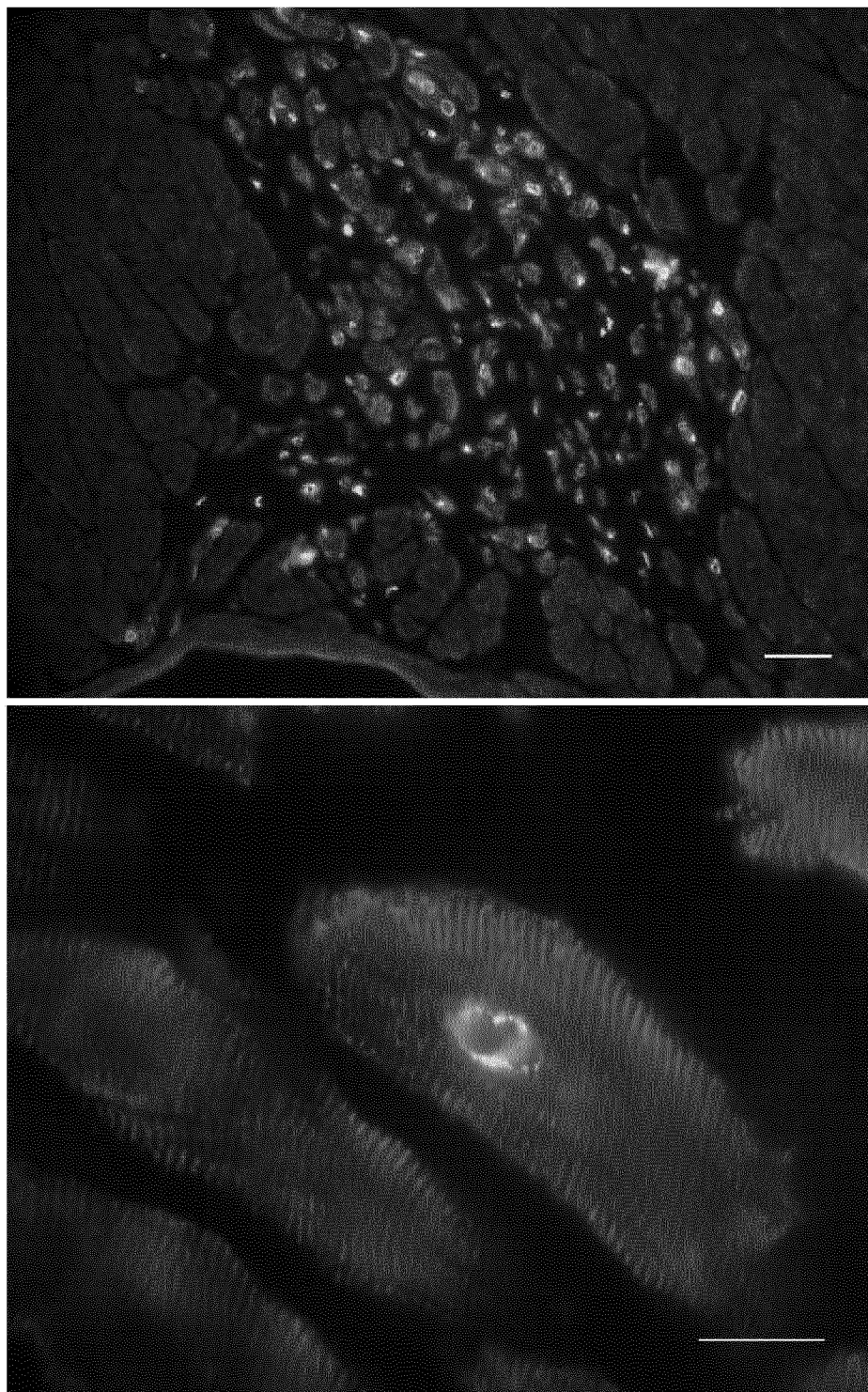


Figure 13

■ Connexin-43
■ Lamin A/C
■ α -Actinin

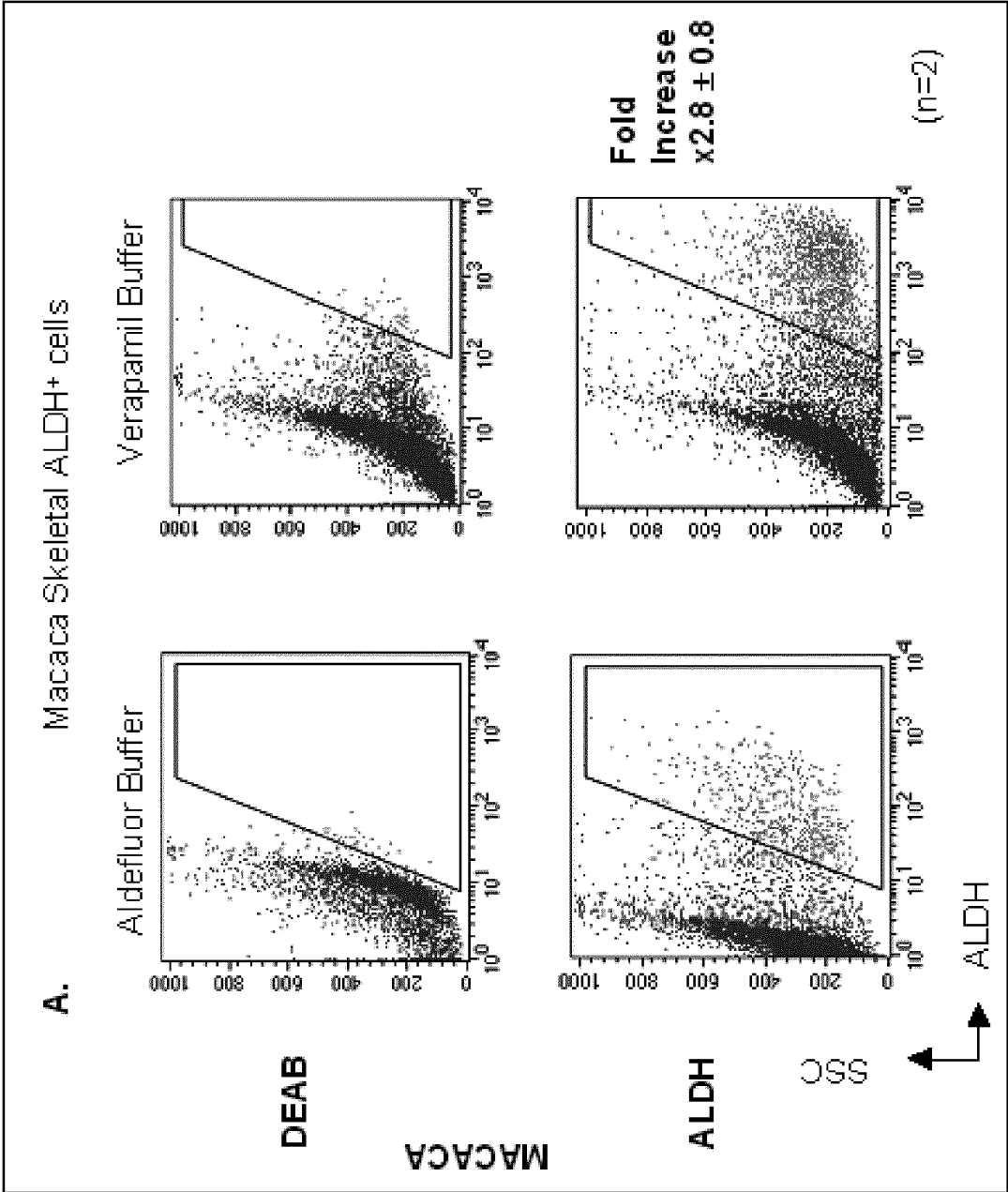


Figure 14

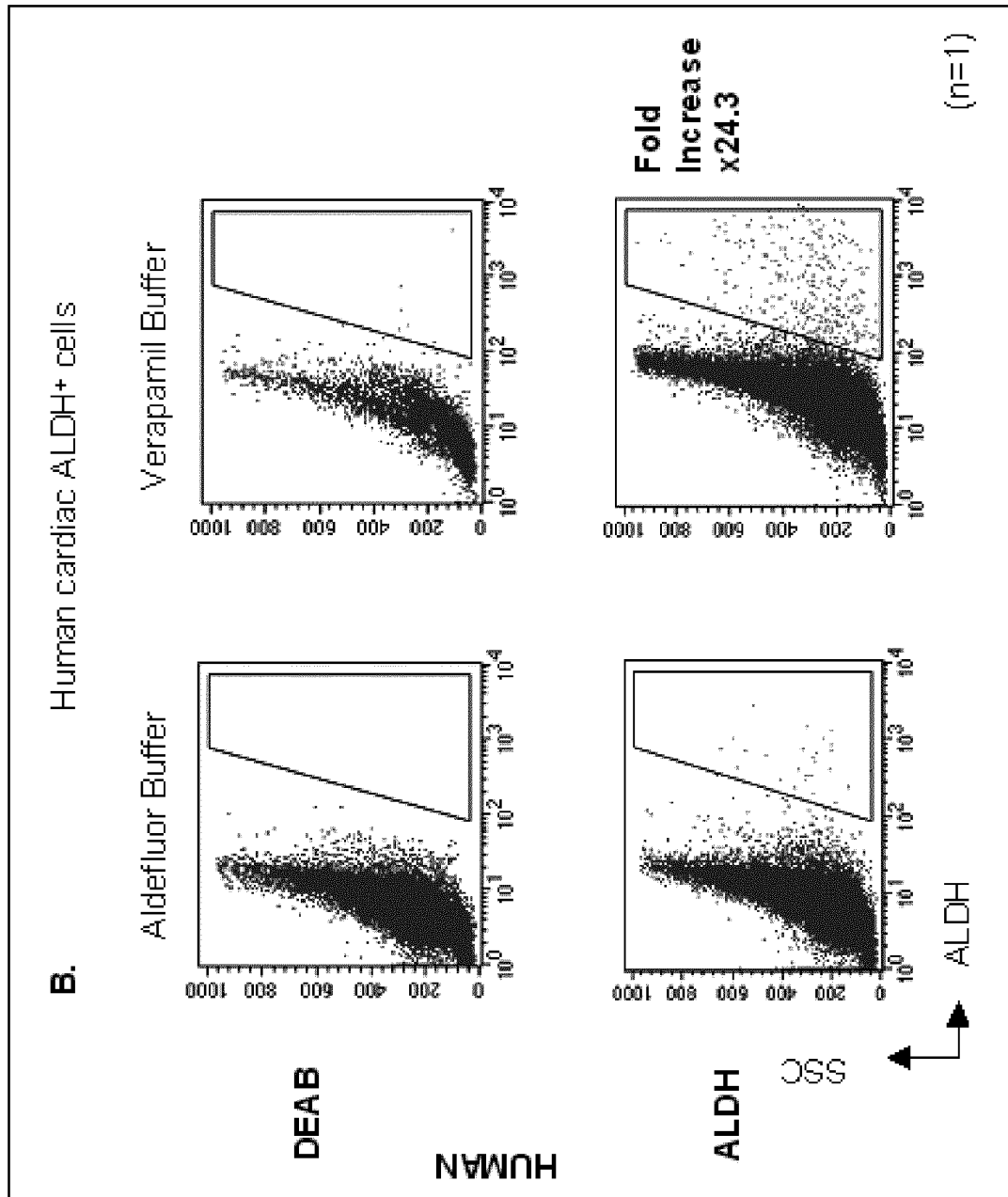


Figure 14 (cont.)

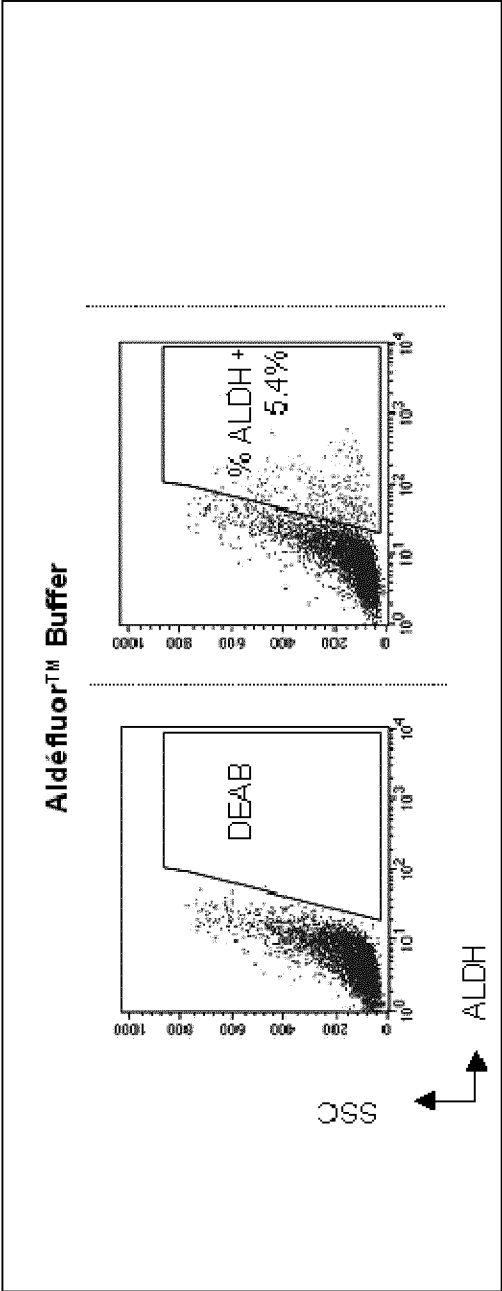


Figure 15

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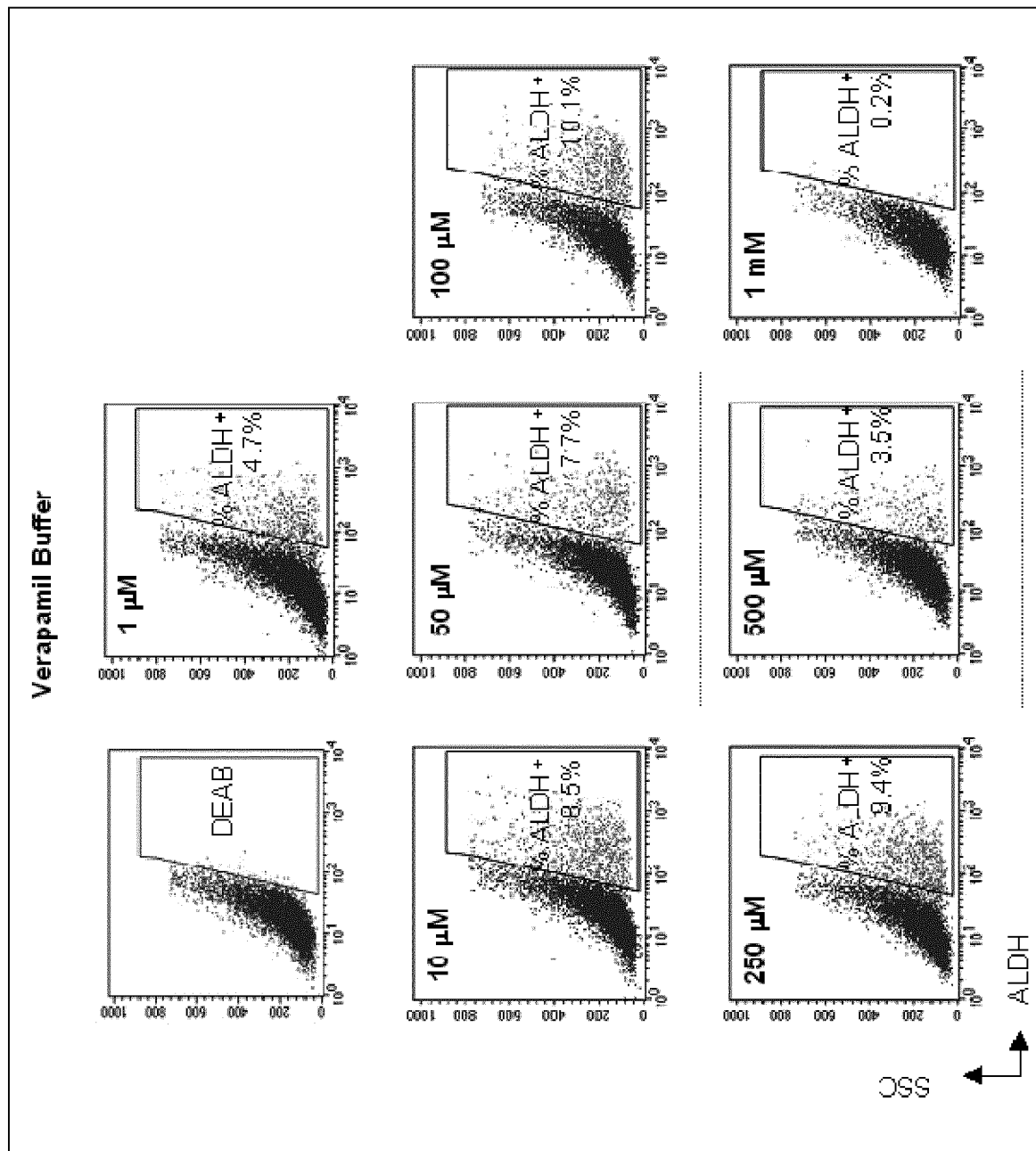


Figure 15 (cont.)

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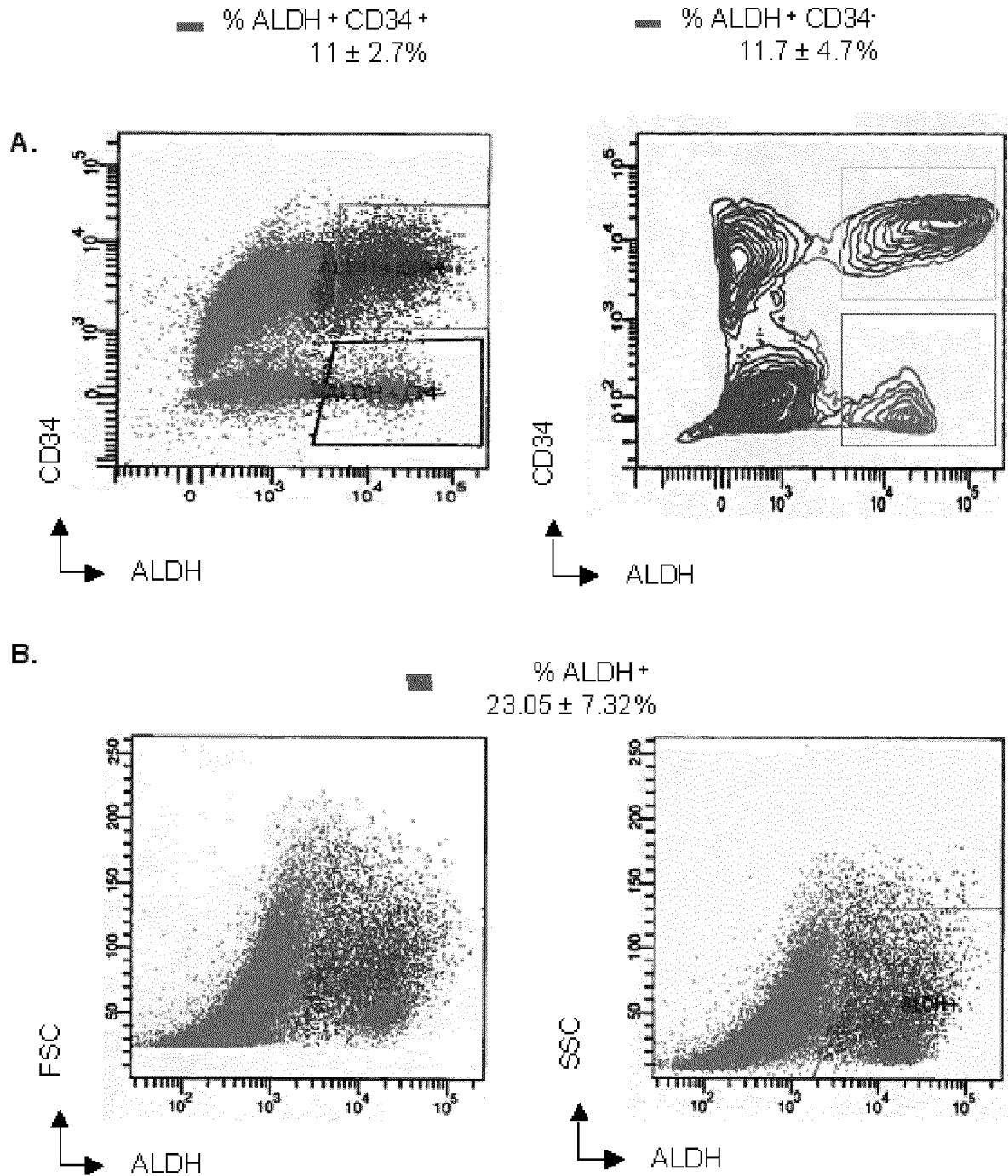


Figure 16

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/079265

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/077
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KARINE VAUCHEZ ET AL: "Aldehyde Dehydrogenase Activity Identifies a Population of Human Skeletal Muscle Cells With High Myogenic Capacities", MOLECULAR THERAPY, vol. 17, no. 11, 8 November 2009 (2009-11-08), pages 1948-1958, XP055122686, ISSN: 1525-0016, DOI: 10.1038/mt.2009.204	1-3
Y	the whole document page 1952, column 3, last paragraph - page 1954, column 3 page 1956, paragraph 3 ----- -/--	5,6



Further documents are listed in the continuation of Box C.



See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

3 March 2015

Date of mailing of the international search report

10/03/2015

Name and mailing address of the ISA/

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Authorized officer

Zuber Perez, C

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/079265

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 206 774 A1 (GENZYME CORP [US]; INST NAT SANTE RECH MED [FR]) 14 July 2010 (2010-07-14) cited in the application	1-3
Y	the whole document page 36; table 3 figure 8	5,6
X	----- TETSURO TAMAKI ET AL: "Clonal Differentiation of Skeletal Muscle-Derived CD34 - /45 - Stem Cells Into Cardiomyocytes In Vivo", STEM CELLS AND DEVELOPMENT, vol. 19, no. 4, 1 April 2010 (2010-04-01), pages 503-512, XP055123109, ISSN: 1547-3287, DOI: 10.1089/scd.2009.0179 the whole document	1-3
X	----- INVERNICI ET AL: "Human adult skeletal muscle stem cells differentiate into cardiomyocyte phenotype in vitro", EXPERIMENTAL CELL RESEARCH, ACADEMIC PRESS, US, vol. 314, no. 2, 7 December 2007 (2007-12-07), pages 366-376, XP022382703, ISSN: 0014-4827, DOI: 10.1016/J.YEXCR.2007.08.006	1-4,7, 9-12
Y	the whole document figures 2-3, 5	5,6
X	----- JASON TCHAO ET AL: "Engineered Human Muscle Tissue from Skeletal Muscle Derived Stem Cells and Induced Pluripotent Stem Cell Derived Cardiac Cells", INTERNATIONAL JOURNAL OF TISSUE ENGINEERING, vol. 72, no. 1, 1 January 2013 (2013-01-01), pages 1-15, XP055122501, ISSN: 2314-4408, DOI: 10.1007/s00246-011-9888-9 the whole document page 23; table 1 figures 3-8	1-4,7-12
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/079265

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARC-ESTIENNE ROEHRICH ET AL: "Characterization of Cardiac-Resident Progenitor Cells Expressing High Aldehyde Dehydrogenase Activity", BIOMED RESEARCH INTERNATIONAL, vol. 109, no. 8, 1 January 2013 (2013-01-01), pages 894-15, XP055122567, ISSN: 2314-6133, DOI: 10.1089/scd.2008.0291	1-3, 10-12
Y	the whole document figures 3, 8e	5,6
X	----- WO 2007/070964 A1 (ES CELL INT PTE LTD [SG]; IP ORGANISERS PTY LTD [AU]; DAVIDSON BRUCE P) 28 June 2007 (2007-06-28) the whole document page 3, last paragraph claim 10	10-14
X	----- GROS ALENA ET AL: "Verapamil enhances the antitumoral efficacy of oncolytic adenoviruses", MOLECULAR THERAPY, NATURE PUBLISHING GROUP, GB, vol. 18, no. 5, 1 May 2010 (2010-05-01), pages 903-911, XP009133788, ISSN: 1525-0024 "plaque assay"; page 910 -----	15,16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/079265

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
EP 2206774	A1	14-07-2010	EP 2206774 A1	14-07-2010
			US 2011008296 A1	13-01-2011

WO 2007070964	A1	28-06-2007	AU 2006326853 A1	28-06-2007
			GB 2447191 A	03-09-2008
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			US 2009202498 A1	13-08-2009
			US 2014193909 A1	10-07-2014
			WO 2007070964 A1	28-06-2007
