CD99R AS MARKER FOR ALPHA AND BETA CELLS

TECHNICAL FIELD

The present invention relates to the field of cell culture and cell therapy, more particularly to methods of enriching and analyzing cultures of glucagon producing alpha cells and insulin producing beta cells.

BACKGROUND TO THE INVENTION

Type 1 diabetes is an autoimmune disease which leads to the destruction of insulin producing beta cells. Beta cells play a crucial role in maintaining glucose homeostasis by producing and secreting hormone insulin in response to blood glucose levels. Loss of beta cells in type 1 diabetes causes insulin deficiency which results in hyperglycemia. Patients with type 1 diabetes are primarily dependent on exogenous insulin injections to manage their blood glucose levels. However, exogenous insulin injections do not provide a solution for physiological glucose homeostasis. Previous studies have shown that pancreatic islet transplantations provide better glycemic regulation and insulin independence in type 1 diabetic patients. However, the lack of donors, procurement of pancreas, yield of islets and immune rejection are major limitations with the islet transplantation (Shapiro et al., 2017). An alternative approach to overcome these limitations is the use of human embryonic stem cell (hESC) derived or induced pluripotent stem cell (iPSC) derived islet cells including insulin secreting beta cells and glucagon secreting alpha cells. Although differentiation protocols have been developed by several labs to generate stem cell derived islet cells, the composition and purity of stem cell derived islet cells remains a challenge in all differentiation protocols and, as an additional complication, is cell line dependent. Stem cell derived islets, from most of the protocols, consists of 30% to 50% unwanted/unknown cells apart from the beta and alpha cells (Balboa et al., 2022; Pagliuca et al., 2014; Rezania et al., 2014; Velazco-Cruz et al., 2019). In a cell therapy product, it is important to not only know which cell types are present but also to reach as high purity of the therapeutically beneficial cell type as possible for the best therapeutic potential. Unwanted cells may be detrimental to the intended islet function and at worst may have the potential risk of teratoma formation after transplantation. Therefore, strategies to get rid of these unwanted cells are important for clinical applications.

The Xg blood group protein CD99 has been described as human beta cell surface marker and used to purify human pancreas fractions with magnetic beads (Martens et al., 2018). In the context of human embryonic stem cell differentiation, CD99 has been used as a marker for definitive endoderm stage (EP4039798 A1). These studies relate to the more broadly expressed CD99 isoform.

Previous studies have demonstrated that CD99R isoform expression is very specific and restricted to subsets of T cells, NK cells and monocytes. In contrast, the CD99 isoform is expressed broadly in several cell types (Bernard et al., 1984; Gelin et al., 1991; Lanza et al., 1994).

CD49a is a well-known cell surface marker for cell stem derived beta cells. A previous study has demonstrated that stem cell derived beta cells can be enriched by CD49a positive cell sorting. CD49a sorted islets consist of up to 80% beta cells and around 5% enterochromaffin cells. However, the limitation with CD49a enriched islets is that they are deficient in glucagon producing alpha cells (Veres et al., 2019).

An object of the present invention is the provision of alternative and/or improved methods and/or means for enriching glucagon producing alpha cells and/or insulin producing beta cells and analyzing populations of such cells.

DEFINITIONS

The term **glucagon producing alpha cells** refers to a type of cells normally found in the islets of Langerhans, responsible for the production of glucagon, a peptide hormone regulating glucose homeostasis. Glucagon production starts with preproglucagon, which contains a signal peptide removed by signal peptidase, thus forming proglucagon (160 aa in humans). Proglucagon is converted by proprotein convertase 2 to glucagon (amino acids 33-61 of preproglucagon). Further products derived from proglucagon produced in intestinal L cells include proglucagon is cleaved to the alternate products glicentin (amino acids 1–69 of proglucagon), glicentin-related pancreatic polypeptide (1–30), oxyntomodulin (33–69), glucagon-like peptide 1 (72–107 or 108), and glucagon-like peptide 2 (126–158). In the present context, the term preferably encompasses all cells producing glucagon, including mature and immature forms of glucagon-producing cells.

The term **insulin producing beta cells** refers to refers to a type of cells normally found in the islets of Langerhans, responsible for the production of insulin. Insulin is produced as pre-proinsulin, which is processed post-translationally to proinsulin and then to insulin. Mature insulin contains disulfide-linked A and B chain fragments of proinsulin. In the processing from proinsulin to insulin, insulin C-peptide, which is not hormonal but can be useful as marker of insulin secretion or production, is released. In the present context, the term preferably encompasses all cells producing mature insulin. In preferred embodiments, the term may also encompass cells that are immature forms of beta-cells that produce insulin although they do not yet process it to mature insulin and may or may not be glucose-responsive. In most preferred embodiments, the term encompasses cells that are glucose-responsive in the sense of being capable of secreting insulin in response to challenge with 20 mM glucose.

The term **CD99** refers to the Xg blood group protein encoded by the CD99 gene with the GenBank Gene ID 4267 (as of 2024-01-19). According to the GenBank RefSeq entry (Mar 2016) associated with this gene, the protein is a cell surface glycoprotein involved in leukocyte migration, T-cell adhesion, ganglioside GM1 and transmembrane protein transport, and T-cell death by a caspase-independent pathway. In addition, the encoded protein may have the ability to rearrange the actin cytoskeleton and may also act as an oncosuppressor in osteosarcoma. The human gene is found in the pseudoautosomal region of chromosomes X and Y and escapes X-chromosome inactivation. There is a related pseudogene located immediately adjacent to this locus. A total of 7 transcripts have been identified originating from the CD99 gene. The protein has been alternatively designated as MIC2, HBA71, MIC2X, MIC2Y and MSK5X in the literature. According to the Human Protein Atlas (online as of 2024-01-19), CD99 the tissue RNA expression has low tissue specificity, and protein expression tissue profile is described as being “Membranous expression most abundant in islets of Langerhans, cells in seminiferous ducts and subsets of lymphoid cells.”

The term **CD99R** refers to a particular isoform of CD99 characterized by a particular epitope having a restricted tissue distribution. The unique tissue-restricted epitope of CD99R was discovered when it was noticed that different monoclonal antibodies to CD99 detected different CD99 isoforms having different tissue distributions (Gelin et al., 1991). CD99R may be characterized by that it is a CD99 protein comprising an epitope expressed on

hematopoietic progenitors, thymocytes, a subset of T cells (such as approximately 30-42% of CD4+ cells and 15-23% of CD8+ cells) and NK cells, but not on B cells, erythrocytes or platelets.

The term "comprising" is to be interpreted as including, but not being limited to.

Numerical values in the specification and claims of this application should be understood to include numerical values which are the same when reduced to the same number of significant figures and numerical values which differ from the stated value by less than the experimental error of conventional measurement technique of the type described in the present application to determine the value.

All ranges disclosed herein are inclusive of the recited endpoint and independently combinable (for example, the range of "from 2 to 10" is inclusive of the endpoints, 2 and 10, and all the intermediate values).

The term "about" can be used to include any numerical value that can vary without changing the basic function of that value. When used with a range, "about" also discloses the range defined by the absolute values of the two endpoints, e.g. "about 2 to about 4" also discloses the range "from 2 to 4." The term "about" may refer to plus or minus 10% of the indicated number.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Schematic diagram showing stepwise differentiation of human pluripotent stem cells into pancreatic islets (A). Flow cytometry plot demonstrating percentage of beta (INS+), alpha (GCG+) and non-alpha/non-beta (INS-/GCG-) cells in stem cell derived islets (B).

Figure 2. Graph demonstrating percentages of insulin and glucagon positive cells in positive fraction of different cell surface markers (A). Graph showing percentage of non-alpha/non-beta cells in positive and negation fraction of different cell surface markers (B). CD49a and CD99 are indicated for reference.

Figure 3. Flow cytometry plot showing beta, alpha and non-alpha/non-beta (INS-/GCG-) cells in stem cell derived islets (A), Isotype control for CD99R (B), CD99R positive cell population (C), CD99R expression in insulin positive cells (D) and glucagon positive cells (E). Flowjo analysis showing different predicted cell populations in CD99R positive fraction (F). CD99R antibody used here was from BD Biosciences (Clone HIT4).

Figure 4. Reference flow cytometry plot showing beta (insulin+), alpha (glucagon+) and non-alpha/non-beta cells in stem cell derived islets (A), Reference Flowjo analysis showing different predicted cell populations in CD99 positive fraction (B).

Figure 5. Flow cytometry plot showing beta (insulin+), alpha (glucagon+) and non-alpha/non-beta cells in stem cell derived islets (A), CD99R positive cell population (B). Flowjo analysis showing different predicted cell populations in CD99R positive fraction (C).

Figure 6. Flow cytometry plot showing beta (insulin+), alpha (glucagon+) and non-alpha/non-beta cells in stem cell derived islets (A), CD99R expression in insulin positive cells (B) and glucagon positive cells (C). Reference flow cytometry plot showing beta, alpha and non-alpha/non-beta cells in stem cell derived islets (D), CD49a expression in insulin positive cells (E) and glucagon positive cells (F).

Figure 7. Flow cytometry plot showing CD99R positive fraction before sorting in stem cell derived islets (A). CD99R positive fraction after sorting (B). CD99R negative fraction after sorting (C). CD99R sorted and reaggregated islets at the end of stage 7 (D). Bar graph summarizing CD99R positive and negative fraction before and after sorting (E).

Figure 8. Flow cytometry plot showing percentage of beta, alpha and non-alpha/non-beta cells in stem cell derived unsorted islets (A). Percentage of beta, alpha and non-alpha/non-beta cells in CD99R sorted islets (B). Bar graph summarizing percentage of beta, alpha and non-alpha/non-beta cells in unsorted and CD99R sorted islets (C). Bar graph showing data of In-vitro glucose stimulated insulin secretion assay for unsorted and CD99R sorted islets (D).

Figure 9. Single cell transcriptome analysis of sorted stem cell derived islets. Uniform Manifold Approximation and Projection (UMAP) demonstrating Seurat clusters of sorted stem cell derived islets.

Figure 10. Bar graph summarizing data for 5 independent experiments (including experiments in Fig 8C, D but with additional replicates) for the percentage of beta, alpha and non-alpha/non-beta cells in unsorted and CD99R sorted islets (A). Bar graph summarizing data for 5 independent experiments for In-vitro glucose stimulated insulin secretion assay for unsorted and CD99R sorted islets (B). Error bars denote standard error of the mean (SEM).

Figure 11. Flow cytometry plot showing beta (insulin+), alpha (glucagon+) and non-alpha/non-beta cells (A), CD99R expression in insulin positive cells (B) and glucagon positive cells (C) in stem cell derived islets generated using the Velazco-Cruz et al 2019 protocol.

SUMMARY OF THE INVENTION

The present invention relates to the use of CD99R as marker for identification and enrichment of glucagon producing alpha cells and/or insulin producing beta cells in a mixture of cells. The inventors discovered that a cell surface marker CD99R is selectively expressed in stem cell derived beta and alpha cells, but not in unwanted non-alpha/non-beta cells (see Example 2).

The CD99R as a marker is a particularly useful tool in the generation of alpha or beta cells from pluripotent stem cells for e.g. cell therapy, *in vivo* studies or *in vitro* applications such as drug screening. Magnetic bead sorting with CD99R is effective in depleting unwanted cells from the stem cell derived islets and was able to generate stem cell derived islets up to 90% purity (see Example 3). The novel marker CD99R resulted in markedly lower percentage of non-alpha/non-beta cells in the enriched fraction compared to the known marker CD49a or the major CD99 isoform (Fig 2). The CD99R-enriched islets have both better purity and glucose responsiveness compared to non-sorted cells (see Example 4). Example 5 demonstrates utility of the CD99R marker irrespective of differentiation method (see Fig 11).

The present invention relates to the following items. The subject matter disclosed in the items below should be regarded disclosed in the same manner as if the subject matter were disclosed in patent claims.

1. A method for enriching glucagon producing alpha cells and/or insulin producing beta cells in a mixture of cells, comprising:
    a. labeling a cell surface marker in a starting mixture of cells with a label; and
    b. separating the cells that are positive for the cell surface marker from cells that are negative for the cell surface marker to obtain a final mixture of cells that is more enriched in alpha cells and/or beta cells compared to the starting mixture;
    wherein said cell surface marker is CD99R.
2. The method of item 1, wherein the label is an antibody specific to CD99R, optionally comprising an affinity tag.

3. The method of any one of the preceding items, wherein the separation step is performed using beads having affinity to the label to separate the cells positive for the cell surface marker, preferably magnetic beads.
4. The method of item 3, wherein the separation step involves separating the beads from the unbound cells by gravity, centrifugation, filtration or magnetism, preferably magnetism.
5. The method according to any one of items 1-2, wherein the separating step is performed using a fluorescence-activated cell sorter.
6. The method of any one of the preceding items, wherein the mixture of cells is a culture derived *in vitro* from human pluripotent stem cells.
7. The method of any one of the preceding items, wherein the enrichment results in at least 3-fold reduction in the fraction of cells other than alpha cells and/or beta cells, preferably at least 5-fold reduction, more preferably at least 7-fold reduction, most preferably at least 10-fold reduction.
8. A method for obtaining glucagon producing alpha cells and/or insulin producing beta cells comprising:
    a. Providing human pluripotent stem cells;
    b. Differentiating the pluripotent stem cells *in vitro* to obtain a population of cells comprising alpha cells and/or beta cells;
    c. Enriching the alpha cells and/or beta cells using the method of item 1 or any item dependent thereon.
9. The method of item 8, wherein the enriched cells are cultured further *in vitro* after the enrichment step.
10. A method of treatment of a subject in need thereof, comprising:
    a. Obtaining alpha islet cells and/or beta islet cells with the method of item 8 or any item dependent thereon;
    b. Transplanting the cells to a subject in need thereof.
11. The method according to item 10, wherein the subject suffers from type I diabetes.
12. A cell population obtainable by the method of any one of the preceding items.
13. A method for analyzing an *in vitro* cell culture comprising glucagon producing alpha cells, insulin producing beta cells and/or other cells, comprising quantitating the

number or fraction of cells expressing CD99R, wherein expression of CD99R is indicative of the presence of alpha islet cells and/or beta islet cells.

14. The method of item 13, wherein the cell culture is a culture derived *in vitro* from human pluripotent stem cells.
15. The method of any one of items 13-14, wherein the quantitation is performed by flow cytometry using an antibody specific for CD99R.
16. A use of an antibody specific for CD99R for detecting glucagon producing alpha cells and/or insulin producing beta cells.

The arrangement of the present disclosure into sections with headings and subheadings is merely to improve legibility and is not to be interpreted limiting in any way, in particular, the division does not in any way preclude or limit combining features under different headings and subheadings with each other. All references are hereby incorporated by reference.

DETAILED DESCRIPTION

Methods for enriching alpha or beta cells

In a first aspect, the present invention provides a method for enriching glucagon producing alpha cells and/or insulin producing beta cells in a mixture of cells, comprising:

a. labelling a cell surface marker in a starting mixture of cells with a label; and

b. separating the cells that are positive for the cell surface marker (i.e. positive for the label) from cells that are negative for the cell surface marker (i.e. negative for the label) to obtain a final mixture of cells that is more enriched in glucagon producing alpha cells and/or insulin producing beta cells (preferably both) compared to the starting mixture;

wherein said cell surface marker is CD99R.

Preferably, both glucagon producing alpha cells and insulin producing beta cells are enriched simultaneously.

The label may be an antibody specific to CD99R. The label can also be an affibody, or other affinity reagent, such as affimer, aptamer or monobody, specific to CD99R. The label may comprise an affinity tag and/or a fluorescent tag which may be suitable for Fluorescence-activated cell sorting (FACS) or analysis. In some cases, the same tag may have both affinity

and fluorescent label functions. Suitable tags include phycoerythrin (PE), biotin, FITC, green fluorescent protein (GFP), Alexa-set of fluorescent dyes and the like.

When an unlabeled antibody (or other affinity reagent) specific to CD99R is used as the label, it may be bound by a secondary antibody (or other affinity reagent) able specifically bind to the CD99R label.

Monoclonal CD99R-specific antibodies have been described in academic literature (Bernard et al., 1984; Gelin et al., 1991; Lanza et al., 1994; Shen et al., 1986) and are also available commercially (e.g. clone REA323, cat. no. 130-104-852, Miltenyi Biotech, clone HIT4, cat. no. 251093, Abbiotech, and clone 5F57, cat. No. USBIC2444-17A, avantor VWR). It should be noted that many antibodies to CD99 are likely to detect both the more broadly expressed isoform (CD99) and the isoform comprising the restricted epitope (CD99R), since the two isoforms share many if not most epitopes. In contrast, the CD99R antibodies by definition only bind to the restricted isoform having the unique CD99R epitope. Additional monoclonal antibodies can be generated with standard techniques including but not limited to: providing and optionally isolating CD99 antigen from a source where CD99R is expressed (such as HPB-ALL human peripheral blood leukemia T-cell line (DSMZ no. ACC483) or PBMCs), immunizing mice with the CD99 antigen, generating monoclonal antibodies from the immunized mice and then screening the antibodies with respect to antigen reactivity and tissue reactivity distribution (see CD99 and CD99R in the Definitions section) to select a CD99R-specific clone.

The separation step may be performed using beads having affinity to the label to separate the cells positive for the cell surface marker, preferably magnetic beads. To be clear, this means that cells positive for the marker bind to the beads, which are then separated from unbound cells. The separation step may involve separating the beads from any unbound cells by gravity, centrifugation, filtration, acoustics or magnetism, preferably magnetism. Use of fluorescence activated cell sorting is also possible.

The mixture of cells may be a culture derived *in vitro* from human pluripotent stem cells, such as embryonic stem cells or induced pluripotent stem cells. However, the CD99R as marker is envisioned to be useful in other contexts as well.

The enrichment results in at least 2-fold reduction in the fraction of cells other than alpha cells and/or beta cells, preferably at least 3-fold reduction, more preferably at least 5-fold

reduction, even more preferably at least 7-fold reduction, most preferably at least 10-fold reduction.

In general, any delta-cells present in the mixture of cells are not significantly depleted in the enrichment utilizing CD99R, apart from unavoidable losses from cell handling. It is preferred that the enrichment step results in retention of at least 50%, more preferably at least 70%, even more preferably at least 80%, and most preferably at least 90% of the delta-cells.

Methods for obtaining alpha or beta cells

In a second aspect, the present invention provides a method for obtaining glucagon producing alpha cells and/or insulin producing beta cells comprising:

a. Providing human pluripotent stem cells such as embryonic stem cells or induced pluripotent stem cells;

b. Differentiating the pluripotent stem cells *in vitro* to obtain a population of cells comprising glucagon producing alpha cells and/or insulin producing beta cells, preferably both;

c. Enriching the glucagon producing alpha cells and/or insulin producing beta cells (preferably both) using the method of the first aspect.

The enriched cells may be cultured further *in vitro* after the enrichment step. Preferably, both glucagon producing alpha cells and insulin producing beta cells are obtained simultaneously. The enrichment step may be performed when the cells are still at an immature stage, i.e. before the differentiation is complete. Thus, the differentiation and enrichment steps may overlap in time. The cells may be stored frozen at any stage, so the steps (or parts of the steps) may also be separated in time.

The present invention is not particularly limited to specific steps of differentiating the cells. Indeed, several suitable differentiation methods have been described in the literature (Velazco-Cruz et al. 2019, Balboa et al. 2022, Rezania et al. 2014, Pagliuca et al. 2014, etc), and analysis/detection/enrichment using CD99R according to the present invention can be applied regardless of the specific method.

For example, the differentiating may include the following substeps:

1. Pluripotent stem cells (e.g. hESC) are differentiated into definitive endoderm cells. Preferably, for the first 24±12h, the cells are treated with Activin A (TGF-β superfamily cytokine) and a GSK-3 inhibitor such as CHIR99021. For the next 48±12h), cells may be treated with Activin A (100ng/ml).
52. The definitive endoderm cells are differentiated into primitive gut tube cells. Preferably, keratinocyte growth factor (KGF)/Fibroblast growth factor 7 (FGF-7) is used (72±24 h).
3. The primitive gut tube cells are differentiated into posterior foregut cells. Preferably, the cells are cultured (24±12h) in media containing the factors KGF, a hedgehog pathway inhibitor such as SANT-1, a retinoid acid receptor agonist such as retinoic acid, protein kinase C activator such as PDBu and BMP signaling inhibitor such as LDN193189.
4. The posterior foregut cells are differentiated into pancreatic progenitor cells. Preferably, the cells are cultured (for 72±24 h) in media comprising KGF, EGF, Nicotinamide, activin A, a hedgehog pathway inhibitor such as SANT-1, a retinoid acid receptor agonist such as retinoic acid, protein kinase C activator such as PDBu and BMP signaling inhibitor such as LDN193189.
155. The pancreatic progenitor cells are differentiated into endocrine progenitor cells. Preferably, they are cultured in media containing a hedgehog pathway inhibitor such as SANT-1, a retinoid acid receptor agonist such as retinoic acid, BMP signaling inhibitor such as LDN193189, TGF-β type I receptor kinase (ALK5) inhibitor such as ALK5i II, a thyroid hormone receptor agonist such as GC1, a Notch pathway inhibitor such as a γ-secretase inhibitor (e.g. GSI-XX) and betacellulin for 5±2 days. At the end of day 4 cells may be dissociated and single cell suspension prepared. These single cells may be seeded into suspension conditions (e.g. ultra-low attachment plates, spinning flasks, bioreactors or the like) and cultured for an additional 24±12h in the presence of the above-mentioned factors and a ROCK-inhibitor such as H1152.
256. The endocrine progenitor cells are differentiated into stem cell derived islets comprising glucagon producing alpha cells and insulin producing beta cells. The cells may be further cultured (the full differentiation may take 3-5 weeks, but the enrichment may be done sooner) in media containing a thyroid hormone receptor agonist such as GC1, a ROCK-inhibitor such as H1152, antioxidants such as N-acetyl cysteine and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

Needless to say, the use of CD99R marker is applicable to other protocols, as well.

Methods of treatment

In a third aspect, the present invention provides a method of treatment of a subject in need thereof, comprising:

a. Obtaining glucagon producing alpha cells and/or insulin producing beta cells with the method of the second aspect;

b. Transplanting the cells to a subject in need thereof.

The subject may suffer from type I diabetes. Preferably, both glucagon producing alpha cells and insulin producing beta cells are obtained simultaneously.

In a fourth aspect, the present invention provides a cell population obtainable by the method of the first or the second aspects, comprising glucagon producing alpha cells and/or insulin producing beta cells. The cell population may be for use in therapy, particularly for the treatment of type I diabetes via cell transplantation.

Use of CD99R for analysis and detection of alpha or beta cells

In a fifth aspect, the present invention provides a method for analyzing an *in vitro* cell culture comprising glucagon producing alpha cells, insulin producing beta cells and/or other cells, comprising quantitating the number or fraction of cells expressing CD99R, wherein expression of CD99R is indicative of the presence of glucagon producing alpha cells and/or insulin producing beta cells (preferably both).

The cell culture may be a culture derived *in vitro* from human pluripotent stem cells, such as embryonic stem cells or induced pluripotent stem cells.

The quantitation may be performed by flow cytometry using an antibody or other label specific for CD99R, such any label described for the first aspect.

In a sixth aspect, the present invention provides a use of an antibody specific for CD99R for detecting glucagon producing alpha cells and/or insulin producing beta cells (preferably both). The detection may be in a mixture of cells, particularly a population of cells obtained by differentiating pluripotent stem cells *in vitro* to obtain a population of cells comprising glucagon producing alpha cells and/or insulin producing beta cells. The antibody may be as described for the first aspect.

EXAMPLES

The following examples are not to be regarded as limiting. For further information on the experimental details, the skilled reader is directed to a separate section titled Materials and Methods.

Example 1: Stem cell differentiation into pancreatic islet cells

For differentiation, human embryonic stem cells (hESCs) were seeded on to laminin 521 (LN521) coated plates at a density of 17000 cells per cm^2 and cultured in NutriStem media for 96 hours. Differentiation was carried out by following a step wise (6 stages) differentiation protocol (Figure 1A). At the end of stage 6, stem cell derived islets contain approximately 40% insulin producing beta cells, 19% glucagon producing alpha cells and 33% non-alpha/non-beta cells (Figure 1B).

Example 2: CD99R expression is specific to stem cell derived beta and alpha cells

Cell surface marker screening (BD Biosciences) was performed to identify specific cell surface markers for insulin and glucagon positive beta and alpha cells. We performed Flowjo analysis to check the proportion of alpha (glucagon positive) and beta cells (insulin positive) within positive fraction of different cell surface markers. We found that CD99R positive fraction contains a high percentage of insulin and glucagon positive cells (Fig 2A). We also found a high proportion of insulin positive cells in a previously reported marker CD49a. However, the percentage of glucagon positive cells was significantly low in CD49a positive fraction in compared to CD99R positive fraction (Fig 2A). Moreover, there was a significantly smaller number of non-alpha/non-beta cells present in CD99R positive fraction in compared to CD49a positive fraction (Figure 2B).

Through the screen we found CD99R to be selectively expressed within stem cell derived beta and alpha cells (Figure 3A-3E). Gating for the CD99R positive fraction increased the beta and alpha cell populations to about 59% and about 23% respectively while reducing the fraction of non-alpha/non-beta cells from about 38% to about 8% (Figure 3F). This is in stark contrast to the most common variant CD99 which was broadly expressed on most of the cells. The CD99 positive fraction also did not eliminate the non-alpha/non-beta cells in a manner comparable to the selective CD99R (Figure 4B) uncovering an unexpected isotype specific expression in stem cell derived islets. We next validated CD99R expression in stem cell derived islets using a different antibody (clone REA323) from Miltenyi Biotec also (Figure 5). These

results demonstrate that CD99R is a specific and positive marker for stem cell derived beta and alpha cells. Further, we compared CD99R expression with a known beta cell marker CD49a in stem cell derived islets. It was found that CD99R expression was specific to both stem cell derived alpha and beta cells (Figure 6A-Figure 6C). While CD49a expression was limited to only stem cell derived beta cells (Figure 6D-Figure 6F).

Example 3: MACS sorting with CD99R to deplete unwanted cells from the stem cell derived islets

At the end of stage 6 differentiation (at day 36), stem cell derived islets were dissociated into single cell suspension using accutase enzyme. Anti-human CD99R PE antibody was added to single cell suspension and incubated for 10 minutes at 4°C. Anti-PE microbeads were then added, and incubation was performed at 4°C for 15 minutes. Mini-MACS column preparation and magnetic sorting was carried out as per manufacturer's protocol. After sorting, CD99R positive fraction showed 90% purity (Figure 7B, 7E). The CD99R positive fraction was seeded into ultra-low attachment 6 well plate (3 million cells per well) and cultured for 3 days in stage 6 medium followed by 7 days in stage 7 medium (Figure 7).

Example 4: CD99R sorted islets have better purity and glucose responsiveness

At the end of stage 7, islets were analyzed by flow cytometry. CD99R sorted islets have better purity and significantly reduced number of unwanted cells in comparison to islets without sorting. CD99R sorted islets consist of approximately 66% insulin producing beta cells, 26% glucagon producing alpha cells and 7% unknown/unwanted cells (Figure 8B, 8C): Figure 10A represents additional replicates (5 independent experiments) including the data in Fig 8. In contrast, islets without sorting contain approximately 48% beta cells, 13% alpha cells and 36% unknown/unwanted cells (Figure 8A, 8C, 10A). Further, single cell transcriptome analysis showed that sorted SC-islets are highly pure. They contain approximately 80% beta cells, 17% alpha cells and just 1.4% off-target/unwanted cells (enterochromaffin cells) (Figure 9).

Moreover, islets with CD99R sorting and without sorting were compared for glucose responsiveness via in-vitro glucose stimulated insulin secretion assay. In response to 20mM glucose, CD99R sorted islets have significantly increased c-peptide release compared to standard islets without sorting (Figure 8D). Similarly, significantly higher levels of c-peptide release were observed for CD99R sorted islets after KCl challenge (Figure 8D, 10B).

Example 5: Utility of CD99R using alternative differentiation protocol

To demonstrate broad utility, we checked the expression of CD99R along with insulin and glucagon in stem cell derived islets generated from using a different protocol (Velazco-Cruz et al 2019). In these stem cell derived islets (Figure 11A) also we observed similar results that CD99R expression was specific to both stem cell derived alpha and beta cells (Figure 11B-Figure 11C).

MATERIALS AND METHODS

Stem cell differentiation into pancreatic islets:

Human pluripotent stem cells (hPSCs) were cultured in NutriStem media. For differentiation, stem cells (17000 cells per cm^2) were seeded on to laminin 521 coated dish and cultured for 96 hours. Differentiation was performed in a stepwise manner using following differentiation media.

Stage 1 (3 days): S1 media + 100 ng/ml Activin A, 5 µM CHIR99021 (6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile) for 1 day. S1 media + 100 ng/ml Activin A for 2 days.

Stage 2 (3 days): S2 media + 50 ng/mL keratinocyte growth factor (KGF).

Stage 3 (1 day): S3 media + 50 ng/mL KGF, 0.25 µM SANT-1 (*N*-[(3,5-dimethyl-1-phenyl-1*H*-pyrazol-4-yl)methylene]-4-(phenylmethyl)-1-piperazinamine), 2 µM RA (Retinoic acid), 500 nM PDBu (Phorbol 12,13-dibutyrate), and 200 nM LDN193189 (4-[6-[4-(1-Piperazinyl)phenyl]pyrazolo[1,5-*a*]pyrimidin-3-yl]quinoline dihydrochloride).

Stage 4 (3 days): S3 media + 50 ng/mL KGF, 100 ng/mL EGF (Epidermal growth factor), 10 mM Nicotinamide, 5 ng/mL Activin A, 0.25 µM SANT1, 100 nM RA, 500 nM PDBu, and 200 nM LDN193189.

Stage 5 (5 days): S5 media + 20 ng/mL Betacellulin, 0.25 µM SANT1, 100 nM RA, 100 nM GSI-XX (γ-secretase inhibitor-XX, *N*-[(1*S*)-2-[[(7*S*)-6,7-Dihydro-5-methyl-6-oxo-5*H*-dibenz[*b*,*d*]azepin-7-yl]amino]-1-methyl-2-oxoethyl]-3,5-difluorobenzeneacetamide), 10 µM Alk5i II (ALK5 Inhibitor II, 2-(3-(6-Methylpyridine-2-yl)-1*H*-pyrazol-4-yl)-1,5-naphthyridine), 1 µM GC-1 (Sobetirome, 2-[4-[[4-Hydroxy-3-(1-methylethyl)phenyl]methyl]-3,5-dimethylphenoxy]acetic acid), and 100 nM LDN193189 for 4 days. After 4 days, cells were dissociated using accutase enzyme and cultured as suspension (1.5 million cells per ml) in

following media for 1 day: S5 media + 10 µM H1152 (Rho-kinase inhibitor, (*S*)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-hexahydro-1*H*-1,4-diazepine dihydrochloride), 20 ng/mL Betacellulin, 0.25 µM SANT1, 100 nM RA, 100 nM GSI-XX, 10 µM Alk5i II, 1 µM GC-1, and 100 nM LDN193189.

Stage 6 (22 days): S6 media + 10 µM H1152, 1 µM GC-1, 10 µM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 1 mM N-acetyl cysteine. Stage 7 (7 to 10 days): S7 media + 1 µM GC-1, 10 µM Trolox and 1 mM N-acetyl cysteine.

S1 media: MCDB131 + 2.5 mM D-(+)-Glucose + 25 mM NaHCO3 + 2 mM Glutamax + 0.5% Pen/Strep + 0.2-0.5% FAF-BSA.

S2 media: MCDB131 + 2.5 mM D-(+)-Glucose + 25 mM NaHCO3 + 2 mM Glutamax + 0.5% Pen/Strep + 0.2-0.5% FAF-BSA + 0.25 mM Vitamin C.

S3 media: MCDB131 + 2.5 mM D-(+)-Glucose + 25 mM NaHCO3 + 2 mM Glutamax + 0.5% Pen/Strep + 0.5% FAF-BSA + 0.25 mM Vitamin C + 1:200 ITS-X.

S5 media: MCDB131 + 2.5 mM D-(+)-Glucose + 25 mM NaHCO3 + 2 mM Glutamax + 0.5% Pen/Strep + 0.5% FAF-BSA, 1:200 ITS-X, 20 mM Glucose, 10 µM ZnSO4, and 10 µg/mL Heparin.

S6 media: CMRL-1066 + 14 mM NaHCO3 + 2 mM Glutamax + 0.5% Pen/Strep + 1% FAF-BSA + 1:200 ITS-X + 1x NEAA + 14.45 mM Glucose + 10 µM ZnSO4 + 10 µg/mL Heparin + 1 mM N-acetyl cysteine + 10 µ M Trolox

S7 media: CMRL-1066 + 14 mM NaHCO3 + 2 mM Glutamax + 0.5% Pen/Strep + 1% FAF-BSA + 1x NEAA + 14.45 mM Glucose + 10 µM ZnSO4 + 10 µg/mL Heparin + 1 mM N-acetyl cysteine + 10 µM Trolox

Cell surface marker screening:

Cell surface marker screening was performed by using BD Lyoplate Human cell surface marker screening panel. Staining for cell surface markers was performed as per manufacturer's instructions. PE conjugated goat anti-mouse Ig and PE conjugated goat anti-rat Ig secondary antibodies were used as detection reagents. After surface marker staining, cells were fixed and permeabilized with cytofix/cytoperm reagent (BD). It was followed by intracellular staining with Alexa fluor 647 mouse anti Insulin (BD) and BV421 mouse anti Glucagon (BD) antibodies. Samples were analyzed in Cytoflex flow cytometry instrument. Data was analyzed using Flowjo software.

CD99R positive cell sorting:

SC-islets were dissociated in single cell suspension using accutase. Cells were stained with PE conjugated CD99R antibody (Miltenyi Biotec). Anti-PE microbeads ultrapure was used for magnetic labelling and sorting was performed using MS column as per manufacturer's instructions. After sorting, CD99R positive fraction was further cultured for 10 days.

Static GSIS (glucose stimulated insulin secretion) assay:

Twenty to thirty SC-islets were incubated overnight in S7 media with low glucose (5 mM final concentration). Next day, SC-islets were washed twice with Krebs buffer and then equilibrated for 2 hours in Krebs buffer containing 2 mM glucose. After this SC-islets were washed once with Krebs buffer and incubated again in Krebs buffer containing 2 mM glucose for 30 minutes. After 30 minutes incubation supernatant was collected and stored. Next, SC-islets were treated step by step with Krebs buffer containing 20 mM glucose, again Krebs buffer containing 2 mM glucose and in last with Krebs buffer containing 2 mM glucose and 30 mM KCl. After each step (treatment) the supernatant was saved. After KCl challange (Krebs buffer containing 2 mM glucose and 30 mM KCl) islets were dissociated into single cells and number of cells were counted. Human c-peptide ELISA was performed to determine c-peptide levels in supernatants collected from each step.

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CLAIMS

1. A method for enriching glucagon producing alpha cells and/or insulin producing beta cells in a mixture of cells, comprising:
    a. labeling a cell surface marker in a starting mixture of cells with a label; and
    b. separating the cells that are positive for the cell surface marker from cells that are negative for the cell surface marker to obtain a final mixture of cells that is more enriched in glucagon producing alpha cells and/or insulin producing beta cells compared to the starting mixture; wherein said cell surface marker is CD99R.
2. The method of claim 1, wherein the label is an antibody specific to CD99R, optionally comprising an affinity tag.
3. The method of any one of the preceding claims, wherein the separation step is performed using beads having affinity to the label to separate the cells positive for the cell surface marker, preferably magnetic beads.
4. The method of claim 3, wherein the separation step involves separating the beads from unbound cells by gravity, centrifugation, filtration or magnetism, preferably magnetism.
5. The method according to any one of claims 1-2, wherein the separating step is performed using a fluorescence-activated cell sorter.
6. The method of any one of the preceding claims, wherein the mixture of cells is a culture derived *in vitro* from human pluripotent stem cells.
7. The method of any one of the preceding claims, wherein the enrichment results in at least 3-fold reduction in the fraction of cells other than glucagon producing alpha cells and/or insulin producing beta cells.
8. A method for obtaining glucagon producing alpha cells and/or insulin producing beta cells comprising:
    a. Providing human pluripotent stem cells;
    b. Differentiating the pluripotent stem cells *in vitro* to obtain a population of cells comprising glucagon producing alpha cells and/or insulin producing beta cells;
    c. Enriching the glucagon producing alpha cells and/or insulin producing beta cells using the method of any one of claims 1-7.

9. The method of claim 8, wherein the enriched cells are cultured further *in vitro* after the enrichment step.
10. A method of treatment of a subject in need thereof, comprising:
    a. Obtaining glucagon producing alpha cells and/or insulin producing beta cells with the method of any one of claims 8-9;
    b. Transplanting the cells to a subject in need thereof.
11. The method according to claim 10, wherein the subject suffers from type I diabetes.
12. A cell population comprising glucagon producing alpha cells and/or insulin producing beta cells, obtainable by the method of any one of the preceding claims.
13. A method for analyzing an *in vitro* cell culture comprising glucagon producing alpha cells, insulin producing beta cells and/or other cells, comprising quantitating the number or fraction of cells expressing CD99R, wherein expression of CD99R is indicative of the presence of glucagon producing alpha cells and/or insulin producing beta cells.
14. The method of claim 13, wherein the cell culture is a culture derived *in vitro* from human pluripotent stem cells.
15. The method of any one of claims 13-14, wherein the quantitation is performed by flow cytometry using an antibody specific for CD99R.
16. A use of an antibody specific for CD99R for detecting glucagon producing alpha cells and/or insulin producing beta cells.