phenotype and function but also allows storage and maintenance of cells while batch release studies are performed before transplanting these cells in a subject.

The inventors have found that the method comprising the steps of dissociating, cryopreserving and re-aggregating endocrine cells co-expressing NKX6.1 and C-peptide, or endocrine progenitor cells co-expressing NKX2.2 and NKX6.1, allows to:

- enrich cell aggregates with endocrine cells co-expressing NKX6.1 and C-peptide;
- reduce the non-endocrine cells (i.e. NKX6.1/C-pep/Glu negative cells) in the cell aggregates;
- reduce cluster heterogeneity and cluster size, which reduces variation *in vivo*;
- reduce and control batch-to-batch variation;
- store and maintain endocrine and endocrine progenitor cells;
- separate the steps of cell production from transplantation, allowing batch tests to be performed.

SUMMARY OF THE INVENTION

In one aspect, the present disclosure refers to a method of cryopreserving pancreatic endocrine cell aggregates derived in vitro from stem cells comprising the following steps: (i) dissociating said pancreatic endocrine cell aggregates into single cells; (ii) treating said single cells with cryopreservation medium and lowering temperature to obtain cryopreserved single cells. In another aspect, the present disclosure refers to a method of enriching endocrine cell aggregates derived *in vitro* from stem cells with endocrine cells co-expressing NKX6.1 and C-peptide or with endocrine progenitor cells co-expressing NKX2.2 and NKX6.1 said method comprising the following steps: (i) dissociating said endocrine cell aggregates into single cells; (ii) treating said single cells with cryopreservation medium and lowering temperature to obtain cryopreserved endocrine cells, (iii) thawing said cryopreserved endocrine cells; and (iv) re-aggregating said endocrine cells obtained after thawing. In another aspect, the present disclosure refers to cryopreserved single endocrine cells co-expressing NKX2.2 and NKX6.1 or co-expressing NKX6.1 and C-peptide. In yet another aspect, the present disclosure refers to cryopreserved single endocrine cells obtained according to the method of cryopreserving as disclosed herein. In yet another aspect, the present disclosure refers to cryopreserved single endocrine cells as disclosed herein for use in the transplantation into a subject or for use in treating diabetes. In still another aspect, the present disclosure refers to re-aggregated endocrine cells comprising at least 50%, preferentially at least 60%, more preferentially at least 70%, even more preferentially at least 80% of endocrine cells co-expressing NKX6.1 and C-peptide or endocrine progenitor cells

co-expressing NKX2.2 and NKX6.1. In a further aspect, the present disclosure refers to re-aggregated endocrine cells obtained according to the method of enriching endocrine cell aggregates as disclosed herein. In still another aspect, the present disclosure refers to re-aggregated endocrine cells as disclosed herein for use in the transplantation into a subject or for use in treating diabetes. In yet another aspect, the present disclosure refers to a composition containing re-aggregated endocrine cells as disclosed herein for use in the transplantation into a subject or for use in treating diabetes or for use as a medicament. In still another aspect, the present disclosure refers to a medicament containing re-aggregated endocrine cells as disclosed herein. In still a further aspect, the present disclosure refers to a device comprising cryopreserved single endocrine cells as disclosed herein, or re-aggregated endocrine cells co-expressing NKX6.1 and C-peptide as disclosed herein, or a composition as disclosed herein, or a medicament as disclosed herein. In another aspect, the present disclosure refers to the use of the cryopreserved single endocrine cells or the re-aggregated endocrine cells as disclosed herein in the manufacture of a medicament for treating diabetes.

The present disclosure provides large scale methods for enriching NKX6.1 and C-peptide co-expressing cell aggregates derived *in vitro* from stem cells. The present method allows enriching cell aggregates derived *in vitro* from stem cells with endocrine cells co-expressing NKX6.1 and C-peptide or co-expressing NKX2.2 and NKX6.1.

The present disclosure provides methods for cryopreserving pancreatic endocrine progenitor cells derived *in vitro* from stem cells comprising the steps of (i) dissociating the cell aggregates into single cells; and (ii) cryopreserving the single cells. The present disclosure is directed to methods to cryopreserved single endocrine progenitor cells co-expressing NKX2.2 and NKX6.1 or single endocrine cells co-expressing NKX6.1 and C-peptide derived *in vitro* from stem cells.

The present disclosure further relates to medical use of the cryopreserved endocrine cells co-expressing NKX6.1 and C-peptide and/or endocrine progenitors cells co-expressing NKX2.2 and NKX6.1 and post cryopreservation *inter alia* in the treatment of type I diabetes.

The present disclosure further relates to thawing and re-aggregating the cryopreserved cells into cell aggregates enriched with NKX6.1 and C-peptide co-expressing cells.

The present disclosure further relates to medical use of re-aggregated endocrine cells co-expressing NKX6.1 and C-peptide or endocrine progenitors cells co-expressing NKX2.2 and NKX6.1 *inter alia* in the treatment of type I diabetes.

The present disclosure provides methods for enriching cell aggregates derived *in vitro* from stem cells with NKX6.1 and C-peptide co-expressing cells while reducing heterogeneity, cluster size and batch to batch variation.

The invention may also solve further problems that will be apparent from the disclosure of the exemplary embodiments.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: Process Overview: Enrichment of NKX6.1 and C-peptide co-expressing cell aggregates

Human embryonic stem cells (hESC) are differentiated *in vitro*, into endocrine progenitor cells co-expressing NKX2.2 and NKX6.1 or endocrine cells co-expressing NKX6.1 and C-peptide using published protocols (WO2015/028614 and WO2017/144695 respectively). At either stage the cells aggregates are dissociated using enzymatic or non-enzymatic digestion. After dissociation, cells are cryopreserved for example by submerging cells in cryopreservation medium and slowly lowering temperature to -80ºC, to obtain cryopreserved cells. These cryopreserved cells are quickly thawed and re-aggregated into cells co-expressing NKX6.1 and C-peptide.

Figure 2: Dissociation, cryopreservation and re-aggregation of endocrine progenitor cells co-expressing NKX2.2 and NKX6.1: Effects on endocrine phenotype *in vitro*

A) Enrichment of endocrine cells after cryopreservation, thawing and re-aggregation at the endocrine progenitor stage.

hESC were differentiated into beta like cells and analysed for the distribution of endocrine and non-endocrine cell populations. For each experiment, cells from the same batch were either differentiated using a protocol without (controls) or with a dissociation, cryopreservation and re-aggregation step.

B) Upper panel: endocrine population is measured by the presence of NKX6.1 and C-peptide co-expression using flow cytometry. Results are presented as % change compared to controls.

Lower panel: de-enrichment of non-endocrine cells are shown by a transcriptional decrease in the non-endocrine markers: AFP, GHRL, KRT18 and KRT 8 when cells were generated using a protocol with a dissociation, cryopreservation and re-aggregation steps. Enrichment of functional endocrine cells are characterised by a transcriptional increase in endocrine markers: GIPR, GLP1R and IAPP when cells were generated using a protocol with a dissociation, cryopreservation and re-aggregation step.

CLAIMS

1. A method of cryopreserving pancreatic endocrine cell aggregates derived *in vitro* from stem cells comprising the following steps:

(i) dissociating said pancreatic endocrine cell aggregates into single cells;

(ii) treating said single cells with cryopreservation medium and lowering temperature to obtain cryopreserved single cells.

2. The method of cryopreserving pancreatic endocrine cell aggregates according to claim 1, wherein said endocrine cells are endocrine cells co-expressing NKX6.1 and C-peptide or endocrine progenitor cells co-expressing NKX2.2 and NKX6.1.

3. A method of enriching endocrine cell aggregates derived *in vitro* from stem cells with endocrine cells co-expressing NKX6.1 and C-peptide or with endocrine progenitor cells co-expressing NKX2.2 and NKX6.1 said method comprising the following steps:

(i) dissociating said endocrine cell aggregates into single cells;

(ii) treating said single cells with cryopreservation medium and lowering temperature to obtain cryopreserved endocrine cells,

(iii) thawing said cryopreserved endocrine cells; and

(iv) re-aggregating said endocrine cells obtained after thawing;

4. The method of enriching endocrine cell aggregates according to claim 3, wherein said endocrine cells of step (i) are endocrine cells co-expressing NKX6.1 and C-peptide or endocrine progenitor cells co-expressing NKX2.2 and NKX6.1.

5. The method of enriching endocrine cell aggregates according to claim 4, wherein when said endocrine cells of step (i) are endocrine progenitor cells co-expressing NKX2.2 and NKX6.1, said method further comprises a step (v) of differentiating said endocrine progenitor cells co-expressing NKX2.2 and NKX6.1 into endocrine cell aggregates co-expressing NKX6.1 and C-peptide.

6. The method according to claims 1 to 5, wherein said stem cells are embryonic stem cells, preferentially human embryonic stem cells.

7. Cryopreserved single endocrine cells co-expressing NKX2.2 and NKX6.1 or co-expressing NKX6.1 and C-peptide.

8. Cryopreserved single endocrine cells obtained according to the method of cryopreserving according to claim 1 or 2.

9. Cryopreserved single endocrine cells according to claim 7 or 8 for use in the transplantation into a subject or for use in treating diabetes.

10. Re-aggregated endocrine cells comprising at least 50%, preferentially at least 60%, more preferentially at least 70%, even more preferentially at least 80% of endocrine cells co-expressing NKX6.1 and C-peptide or endocrine progenitor cells co-expressing NKX2.2 and NKX6.1.

11. Re-aggregated endocrine cells obtained according to the method of enriching endocrine cell aggregates according to anyone of claims 3 to 6.

12. Re-aggregated endocrine cells according to claim 10 or 11 for use in the transplantation into a subject or for use in treating diabetes or for use as a medicament.

13. Composition containing re-aggregated endocrine cells according to claim 10 or 11 for use in the transplantation into a subject or for use in treating diabetes or for use as a medicament.

14. Medicament containing re-aggregated endocrine cells according to claim 12 or 13.

15. A device comprising cryopreserved single endocrine cells according to anyone of claims 7 to 9, or re-aggregated endocrine cells co-expressing NKX6.1 and C-peptide according anyone of claims 10 to 12, or a composition according to claim 13, or a medicament according to claim 14.

16. Use of the cryopreserved single endocrine cells according to claim 7 or 8 or the re-aggregated endocrine cells according to claim 10 or 11 in the manufacture of a medicament for treating diabetes.