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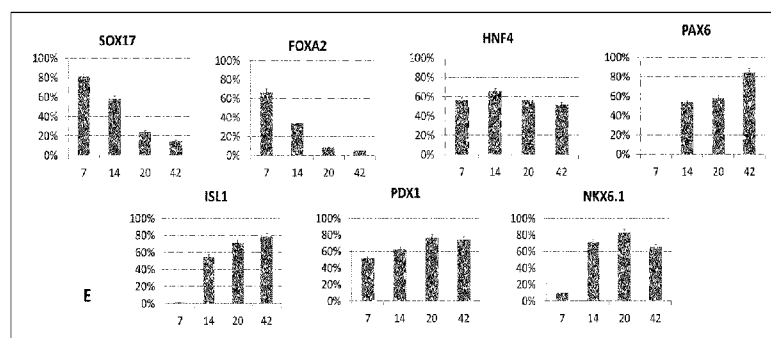
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(54) Title: METHODS FOR THE CONVERSION OF SOMATIC CELLS INTO PANCREATIC-HORMONE SECRETING CELLS

Fig. 3



(57) Abstract: Methods for converting somatic adult cells into pancreatic-hormone secreting cells by a epigenetic DNA modification are provided. Cells obtained according to such methods and treatment methods using such cells are also provided.

DESCRIPTION

METHODS FOR THE CONVERSION OF SOMATIC CELLS INTO
PANCREATIC-HORMONE SECRETING CELLSBackground of the Invention

5 Regenerative medicine requires new cells that
can be delivered to patients for repairing and
renovating degenerated or damaged tissues.

 When such cells are not readily available, two
main strategies have been developed to obtain them.

10 One is directed differentiation, by which
pluripotent cells, exposed to specific cell culture
conditions, designed to mimic natural events, assume
a specific cell fate; the other,
transdifferentiation, also referred to as
15 reprogramming, which enables a fully differentiated
cell type to be converted into another one without
going through an undifferentiated pluripotent stage.

 Induced pluripotent stem cell (iPSC) technology
has shown that the stability of a mature phenotype
20 can be overcome by transforming a somatic cell of any
patient in an unlimited source of autologous
pluripotent cells.

 The elimination of the risk of immune rejection
provided by iPSCs, immediately boosted the clinical
25 potential of directed differentiation.

However, the requirement of permanent integration of viral vectors into the host genome to generate iPSCs, poses a severe limit to their current therapeutic use.

5 This has stimulated the development of several protocols for virus-free iPSC derivation but, at present, these approaches are generally more technically demanding and less efficient and, therefore, have not gained widespread adoption.

10 Similar limitations apply to recent examples of successful transdifferentiation, since the direct conversion of one mature cell type into another has been obtained only through virus-based transfection protocols.

15 Summary of the invention

 The present invention provides virus-free methods for converting somatic adult cells into pancreatic hormone secreting cells, based on epigenetic DNA modification herein named Pancreatic
20 Converted Cells (PCC).

 The present invention also provides methods for converting somatic cells into different cell types by epigenetic DNA modification.

In other embodiments, the invention is directed to cells or a population of cells obtained according to such methods.

In a further embodiment, methods for the
5 treatment of pathologies include use of the cells obtained according to the invention.

In a particular embodiment, an implant comprising cells obtained according to the invention can be used for the control of the level of
10 pancreatic hormones.

In a further embodiment, *in vitro* models for the study of pathologies related to alteration in pancreatic cell function are provided.

According to an additional embodiment, the
15 invention provides methods for identifying the therapeutic effect of a drug on a patient.

Brief description of the figures

Figure 1 shows the morphological changes in adult human skin fibroblasts exposed to 5-azacytidine
20 (5-aza-CR) and subjected to endocrine pancreatic induction; (A) immunolocalization of vimentin (typical fibroblast intermediate filament protein). Scale bar = 200 μ m; (B) Untreated cells (T0) underwent marked morphological changes in response to
25 an 18 h exposure to 5-aza-CR (Post 5-aza-CR). Scale

bar 200 μm ; (C) is a representative pictures of the morphological changes during endocrine pancreatic induction at day 7, 10, 20 and 36;

Figure 2 shows the gene expression changes in adult human skin fibroblasts exposed to 5-aza-CR and subjected to endocrine pancreatic induction;

Figure 3 shows the immunocytochemical localization of endoderm and pancreatic markers during human skin fibroblasts conversion to endocrine pancreatic cells;

Figure 4 represents the morphological and in vitro functional characterization of human PCC;

Figure 5 represents the in vivo functional characterization of human PCC.

15 Detailed description of the invention

Methods are disclosed herein for converting adult cells into pancreatic cells, in particular pancreatic hormone-secreting cells.

Such adult cells may include any type of adult cells.

In certain embodiments, said adult cells may include adult somatic cells.

In other embodiments, said adult somatic cells include adult fibroblasts, including those of a primary line.

In other embodiments, said adult fibroblasts may be adult skin fibroblasts.

Methods of the invention may include the steps of:

5 1) inducing dedifferentiation of said adult cells; and

2) inducing differentiation of said undifferentiated cells to pancreatic hormones-secreting cells.

10 The cells may be human cells.

In an alternative embodiment, the cells may be animal cells.

In particular, said cells can be for instance from: mouse, rat, rabbit, cat, dog, pig, horse and
15 non-human primates.

With reference to step 1) methods of the invention, in particular, may include treatment of the cells with a substance capable of inducing epigenetic modifications and thus increasing cell
20 plasticity.

For such purposes, an inhibitor of the methylation may be used, which can be selected from, for example: 5-azacytidine and any functionally related molecules with histone methylation and
25 demethylation-inhibiting properties such as Bix-

01294, CARM1 inhibitor, daminozide, HMTase Inhibitor V, UNC0224, and the like.

In certain embodiments, an inhibitor of acetylation may be used.

5 For instance, valproic acid or tricostatin may be used in step 1).

In other embodiments of the invention, a combination of epigenetic modifiers may be used, such as valproic acid and 5-azacytidine.

10 According to certain embodiments of the invention, 5-azacytidine is used alone.

As for step 1) dedifferentiation may be performed for a period of from about 6 to about 24 hours.

15 In certain embodiments, it may be performed for about 18 hours.

5-azacytidine may be used at a concentration of about 0.1 to about 5 μ M.

In certain embodiments, it may be performed at a
20 concentration of about 1 μ M.

Alternatively, if 5-azacytidine is used in combination with valproic acid in step 1), a solution of valproic acid at a concentration of about 0.5 - 5 mM for 24-72 hours followed by 5-azacytidine at a

concentration of about 0.5-5 μ M for 12-24 hours may be used.

Before proceeding with step 2), in certain embodiments of the invention, an additional step 1a) may be performed for the recovery of the dedifferentiated (or undifferentiated) cells obtained from step 1).

For said purpose, the cells may be left for a period of at least 3 hours in a medium for embryonic stem cells.

For example, the embryonic stem cell medium of Brevini et al (2009, Stem Cell Rev 5(4): 340-352) may be used.

Before proceeding with the following step, the cells optionally may be rinsed with embryonic stem cell medium.

According to embodiments of the invention, step 2) for differentiating the cells may include a three step-protocol, which comprises:

- 2a) exposing the cells to activin A;
- 2b) exposing the cells to retinoic acid; and
- 2c) exposing the cells to a complex medium.

In step 2a) activin A having a concentration of 1 μ l/ml (or 30 ng/ml) may be added to a pancreatic medium basal.

Said pancreatic medium basal, in particular may comprise:

- DMEM/F12 medium;
- B27 supplement minus Vitamin A;
- 5 - N2 supplement;
- MEM Non-Essential Amino Acids Solution;
- Antibiotic Antimycotic Solution;
- 2-Mercaptoethanol;
- L-Glutamine;
- 10 - Albumin from bovine serum;

According to certain embodiments, the pancreatic medium basal may include the use of:

- B27 supplement minus Vitamin A at a concentration of 2%;
- 15 - N2 supplement at a concentration of 1%;
- MEM Non-Essential Amino Acids Solution at a concentration of 1%;
- Antibiotic Antimycotic Solution at a concentration of 1%;
- 20 - 2-Mercaptoethanol at a concentration of 0.1 mM;
- L-Glutamine at a concentration of 2 mM;
- Albumin from bovine serum at a concentration of 0.05%;

The treatment of step 2a) may last for about 4
25 to 8 days, in certain embodiments 5 to 7 days and in

other embodiments 6 days, with a daily change of the medium.

According to step 2b) retinoic acid may be added to the medium comprising a pancreatic medium basal
5 and activin A 30 ng/ml.

In particular, the retinoic acid may have a concentration of 10 μ M.

With reference to step 2b), in certain embodiments it may be performed for a period of about
10 1 to 3 days and in other embodiments for 2 about days, with a daily medium change.

In step 2c), the cells may be exposed to a complex medium.

Said complex medium, in particular may comprise:

- 15
- pancreatic basal medium;
 - insulin-transferrin-selenium;
 - basic fibroblast growth factor (bFGF);
 - B27 supplement.

Certain embodiments may include the use of:

- 20
- insulin-transferrin-selenium at a concentration of 1% (v/v);
 - basic fibroblast growth factor (bFGF) at a concentration of 10-20 ng/ml;
 - B27 at a concentration of 2% (v/v);

Step 2c) may be continued for about 27 days, in certain embodiments at least 27 days, with a daily change of medium during the first 15 days, then with the medium changed about every other day.

5 In certain embodiments, the duration of the differentiation phase may be at least about 36 days.

Figure 1C shows that during the differentiation process, there can be observed the formation of clusters formed by organized cells (at day 7);
10 distinguishable aggregates can be seen at day 10;
large three-dimensional colonies form at about day 20, while at differentiation, freely floating spherical structures comprising colonies of cells can be observed, which appear as typical pancreatic
15 islets *in vivo* (day 36).

The cells, a population or culture of cells, colonies, aggregates and islets-like formations of cells obtained according to the disclosed methods represent further embodiments of the present
20 application.

According to a particular embodiment, the method described above for converting adult somatic cells, in particular, adult fibroblasts, comprises an additional step 0), which precedes step 1).

During said step 0) the cells may be treated in a fibroblast culture medium, which comprises only about 0.1% to 0.4% of serum, and in certain embodiments only about 0.2 to 0.3% of serum.

5 In such embodiments, serum may be foetal bovine serum.

According to certain embodiments, a content of 0.2% of serum, which is about 1% of the content used for growing and recovering fibroblast cells, may be used.

10 It has been observed that under said conditions (also referred to as "serum starving") the cells maintain the G cellular phase.

Accordingly, the cell population is maintained in said state until step 1) is performed.

15 It has been observed that when the method of the invention is performed on a population of cells that underwent the above treatment, the efficiency of the cell conversion is increased to a surprising extent.

In particular, the efficiency of epigenetic
20 conversion, considering the C-peptide secreting cells, is up to 62.2 +/- 11.7%.

The present invention also provides methods for the treatment of pathologies related to the production or the secretion of pancreatic hormones in a patient

comprising use of cells according to the present description.

For the purpose of the present invention, pancreatic hormones are intended to refer to all the
5 hormones produced by the pancreas, for instance, insulin, C-peptide, somatostatin and glucagon.

In certain embodiments insulin is preferred.

It has been observed that starting from about the 14th day of differentiation, the cells, aggregates and
10 islets obtained according to embodiments of the invention start producing and releasing insulin, C-peptide, somatostatin and glucagon.

In particular, said treatment may include a step wherein the cells, either as a population or
15 aggregates or islets, are grafted or implanted into the body of the patient.

For such purposes, a patient may be a human being or an animal.

In the case of an implant, the cells may be
20 implanted subcutaneously or in the abdominal cavity, for instance on the forearm or elsewhere on the arm, or grafted to the omentum.

In particular, cells may be injected or grafted as pellets or contained in a semi-permeable device
25 according to methods known in the art.

As a result, the production and release of hormones may be advantageously integrated and controlled by the implanted pancreatic hormones secreting cells as a response to blood level of certain hormones or
5 nutrients like glucose.

According to further embodiments, an *in vitro* cellular model which includes the above described cells is provided.

In particular, such a model may be used for
10 studying the pharmacological activity and the toxicity of drugs on pancreatic cells, thereby furnishing useful data for the development of new therapies.

For such purposes, active compounds that may be
15 tested may include anti-diabetic drugs, physiological and pathological levels of nutrients, hormones, metabolic stressors, or any component of the immunological process, etc.

Example 1

20 Culture of skin fibroblasts

Two adult human skin fibroblast primary lines were isolated from two female adults 35 and 49 years old. A skin specimen of approximately 2 mm³ was taken by excision under local anaesthesia from an avascular
25 area of the anterior aspect of the forearm. Cells

were grown to confluence in 60 mm culture dishes in medium DMEM supplemented with 20% fetal bovine serum (FBS). After four passages, fibroblasts were harvested and frozen in liquid nitrogen in several aliquots. Two lines, derived from neonatal foreskin, were commercially available (Gentaur cat. N. SC101A-HFF and ATCC ® cat. N. PCS-201-010). After thawing, cells were grown in standard culture medium consisting of DMEM with 20% FBS (Gibco), 2 mM glutamine (Sigma) and antibiotics (Sigma). Cells were passaged twice a week in a 1:3 ratio.

Example 2

Treatment of skin fibroblasts with 5-azacytidine-CR

Cells were plated in 0.1% gelatin (Sigma) pre-coated 4-well multidish (Nunc) and exposed to 1 μ M 5-aza-CR (Sigma) for 18 h. At the end of the 18 h exposure, cells were rinsed three times.

Incubation of cells with ES cell culture medium was continued for 3 hours (Brevini et al, 2009, Stem Cell Rev 5(4): 340-352).

Pancreatic induction

Cells were cultured in basal pancreatic medium supplemented with 0.1 nM β -mercaptoethanol (Sigma), 2 mM glutamine (Sigma), 1 mM MEM Non-Essential Amino Acids (Gibco) and 0.05% bovine serum albumin (Sigma).

During the first 6 days, medium was supplemented with 30 ng/ml activin A (Biosource). On day 7, 10 μ M retinoic acid (Sigma) was added. Two days later, medium was refreshed and replaced with pancreatic
5 medium basal supplemented with 2% B27 (Invitrogen), 10 ng/ml basic fibroblast growth factor (R&D System) and 1% insulin-transferrin-selenium (Invitrogen) to further encourage differentiation. Medium was refreshed daily. Cells were maintained *in vitro* for a
10 total of 102 days when cultures were arrested.

Cell analysis

Cells were analyzed at the following time points: untreated fibroblasts (T0), after 18 h exposure to 5-aza-CR (Post 5-aza-CR), and then on day
15 7-10-14-20-30-36-42-102 along pancreatic induction.

The results are illustrated in the Figures.

In particular, Figure 1 (A) shows the immunolocalization of vimentin, the typical fibroblast intermediate filament protein. A
20 homogenous cell population at the onset of the experiments was presented. Scale bar = 200 μ m. (B) shows that untreated cells (T0) underwent marked morphological changes in response to an 18 h exposure to 5-aza-CR (Post 5-aza-CR). Fibroblasts changed
25 their typical elongated shape into a round

epithelioid aspect. Cell size was smaller and nuclei became larger and more granular. Scale bar 200 μm .

(C) is a representative depiction of the morphological changes taking place during endocrine pancreatic induction. Cells exposed to activin A gradually organized in clusters (Day 7). In response to the addition of retinoic acid, they rearranged in a reticular pattern and clustered in distinguishable aggregates (Day 10). These formations progressed with time and were further stimulated by B27/bFGF/ITS that led to the recruitment of a growing number of cells, aggregating in large three-dimensional colonies (Day 20). Finally, colonies became spherical structures that tended to detach and float freely in the culture medium, reminiscent of typical pancreatic islets in vitro (Day 36). Scale bar = 400 μm .

Figure 2 shows the gene expression changes in adult human skin fibroblasts exposed to 5-aza-CR and subjected to endocrine pancreatic induction.

In particular, the expression pattern of markers of early (NES, SOX17, FOXA2, HNF4A, HNF1B, ONECUT, PDX1, MAFB) and mature pancreatic precursors (NKX6.1, PAX6, NEUROD, ISL1, MAFA, PCSK1, PCSK2) in untreated fibroblasts (T0), fibroblast exposed to 5-aza-CR

(Post 5-aza-CR) and at different days of pancreatic induction (7 to 102) were analyzed.

Figure 3 shows the immunocytochemical localization of endoderm and pancreatic markers during human skin fibroblast conversion to endocrine pancreatic cells. In particular: (A) shows the immunolocalization of endoderm (SOX17, FOXA2) and primitive gut tube (HNF4) markers in fibroblasts subjected to pancreatic induction for 10 days; (B) the immunolocalization of PAX6 and ISL1, markers of more advanced pancreatic differentiation, on day 30 after exposure to 5-aza-CR; (C) the co-localization of PDX1 and NKX6.1 with C-peptide was also observed on day 30 after exposure to 5-aza-CR; (D) the Western blot immunodetection of SOX17, FOXA2, HNF4, PDX1, NKX6.1, PAX6 and ISL1 in PCC on different days of treatment; (E) the proportion of positive cells for the different molecules during the differentiation process in vitro. Scale bars = 200 μ m.

Figure 4 shows the morphological characterization of human PCC; in particular: (A) the immunostaining of PCC after 36 days of culture reveals a clear signal of C-peptide, somatostatin and glucagon, in positive cells. Scale bar = 200 μ m; (B) represents the Western blot analysis of constitutive

proteins collected at different time of culture.

Insulin, C-peptide, somatostatin, and glucagon were detected from day 14 and steadily increased;

consistently with the absence of its mRNA, ghrelin

5 was not detectable. β -actin was used to check that

equal protein amounts were loaded on each lane; (C)

is the representative output of flow cytometer

analysis showing the efficiency towards β -cell

differentiation measured counting C-peptide labeled

10 cells; (D) is the quantification of C-peptide release

in the culture medium in response to 20mM D-glucose

for 1h at different times of culture. Bars represents

the mean $\pm$ SD of three independent replicates.

Example 3

15 PCC transplantation into diabetic SCID mice

Experimental diabetes was induced in 8-week-old male SCID mice (Harlan) by a single intraperitoneal injection of streptozotocin (STZ; Sigma, 150 mg/kg of body weight) freshly dissolved in 0.1 M of citrate

20 buffer, pH 4.6 (23). Six days after STZ injection the average blood glucose level was $426,30 \pm 38,82$ mg/dl.

Following isoflurane anaesthesia, cells were injected subcutaneously in the shoulder area through a 19-gauge hypodermic needle. Five animals received 5×10^6

25 PCC and 5 received the same number of untreated

fibroblasts. Blood glucose levels were measured using Accu-Chek glucometer (Roche) at 1 week intervals.

Glucose tolerance test

Mice were fasted for 20 h. Glucose was
5 administered as an intraperitoneal injection of a 30% dextrose solution at a dose of 3.0 g per kg body weight. Tail blood glucose levels were measured with Accu-Chek glucometer (Roche) before and 15-30-45-60-75-90 minutes after glucose administration. Data were
10 analyzed with an independent-samples t-test (two-tailed, type 2) using SPSS 19.0, and all values are presented as means $\pm$ standard deviation (SD).

Differences of $p \leq 0.05$ were considered significant

ELISA

15 Blood samples were collected from mice three times at one week interval during PCC engraftment and after its removal. ELISA assays for human C-peptide were performed on serum samples as described by the manufacturer (Mercodia Insulin ELISA cat n.10-1113-
20 01).

Removal of grafted PCC

To verify PCC ability to restore blood glucose homeostasis in STZ-treated mice, PCC engrafts were surgically removed from mice under general
25 anesthesia. Blood glucose levels were then monitored

in the animals for 3 weeks, using Accu-Chek glucometer (Roche).

Immunofluorescence analyses

Pancreases of non-treated and STZ-treated mice
5 and removed grafts were fixed with 10% (wt/vol) formaldehyde for 24 h at 4°C. Tissues were embedded in paraffin and cut into 5µm sections. Slides were deparaffinised and rehydrated. Aspecific sites were blocked with a solution of PBS containing 5% Bovine
10 Serum Albumin and 10% non-immune serum. Samples were incubated overnight at 4°C with antibodies specific for insulin, glucagon and somatostatin (Table S2). Sections were washed three times with PBS and incubated with suitable secondary antibodies
15 (Alexafluor, Invitrogen) for 45 min. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Slides were observed under a Nikon Eclipse 600 microscope.

Figure 5 shows the functional characterization
20 of human PCC; (A) Subcutaneous injection of 5×10^6 PCC in streptozotocin treated SCID mice promptly decreased their glucose blood levels. Glucose levels remained constant up to 133 days. Injection of the same number of untreated fibroblasts did not elicit
25 any effect and the mice died after 4 weeks. Removal

of PCC from streptozotocin-treated mice caused a rapid rise of glycemic values indicating that PCC were the functional source of insulin; (B) intraperitoneal injection of 3 g per kg body weight induced a rise of blood glucose concentration that returned to basal level within 90' minutes both in PCC-engrafted and control mice. The test was repeated 3 times at one week intervals; (C) levels of human insulin in the serum of STZ-treated mice during PCC engraftment and after its removal; (D) immunolocalization of C-Peptide and glucagone in pancreatic islets of control and STZ SCID mice indicate the selective destruction of beta cells (Scale bar 20 μ m). Immunolocalization of C-Peptide, Glucagone and Somatostatin in human PCC cells removed from SCID mice. Merged co-staining demonstrate that each cell produces a single hormone. Scale bar = 50 μ m.

CLAIMS:

1. A method for converting adult cells into pancreatic hormones secreting cell by the epigenetic DNA modification, comprising the steps of:
 - 5 1) inducing dedifferentiation of said cells; and
 - 2) inducing differentiation of said dedifferentiated cells.
2. The method according to claim 1, wherein said adult cells are adult somatic cells.
- 10 3. The method according to claim 1 or 2, wherein said adult somatic cells are adult fibroblasts.
4. The method according to claim 1 or 2 or 3, wherein said adult fibroblasts are adult skin fibroblasts.
5. The method according to any one of claims 1 to 4,
15 wherein said cells are human adult skin fibroblasts.
6. The method according to any one of claims 3 to 5, wherein said fibroblasts are of a primary line.
7. The method according to any one of claims 1 to 4 or 6, wherein said cells are animal cells.
- 20 8. The method according to the preceding claim, wherein said cells are from mouse, rat, rabbit, cat, dog, pig, horse or non-human primates.
9. The method according to any one of the preceding claims, wherein after step 1) and before step 2) it
25 is performed a recovery step 1a).

10. The method according to the preceding claim,
wherein said recovery step 1a) comprises maintaining
the cells in an embryonic stem cell medium.
11. The method according to the preceding claims 9 or
5 10, wherein said step 1a) is performed for a period
of at least 3 hours.
12. The method according to any one of the preceding
claims 9 to 11, wherein said recovery step 1a) is
optionally performed after having rinsed the cells.
- 10 13. The method according to any one of the preceding
claims, wherein before proceeding with the step 1) of
inducing the dedifferentiation, the adult cells are
subjected to a step 0) wherein the cells are
maintained in the G phase.
- 15 14. The method according to the preceding claim,
wherein in said step 0) the adult cells are
maintained under serum starving conditions.
15. The method according to any one of the preceding
claims 3 to 12, wherein before proceeding with step
20 1) of inducing the dedifferentiation, the adult
fibroblasts are subjected to a step 0) of treatment
of the fibroblasts with a fibroblast medium with a
reduced amount of serum.

16. The method according to claim 14, wherein said serum starving conditions include the use of a cell medium with a reduced amount of serum.
17. The method according to the preceding claim 15 or
5 16, wherein said medium comprises an amount of 0.1-0.4% of foetal bovine serum.
18. The method according to the preceding claim 16 or 17, wherein said medium comprises an amount of 0.2-0.3% of foetal bovine serum.
- 10 19. The method according to the preceding claims 16 or 17 or 18, wherein said medium comprises an amount of 0.2 % of serum.
20. The method according to any one of the preceding claims, wherein the dedifferentiation step 1) is
15 performed with an agent capable of inducing an epigenetic modification.
21. The method according to the preceding claim 19 or 20, wherein said agent has histone methylation or demethylation inhibiting activity.
- 20 22. The method according to the preceding claim 19 or 20, wherein said agent is selected in the group comprising: valproic acid, trichostatin, 5-azacytidine, Bix-01294, CARM1 inhibitor, daminozide, HMTase Inhibitor V, UNC0224.

23. The method according to any one of the preceding claims 20 to 22, wherein said agent is 5-azacytidine.

24. The method according to any one of the preceding claims 20 to 23, wherein the dedifferentiation step

5 1) comprises the treatment with 5-azacytidine for a period of 6 to 24 hours.

25. The method according to any one of the preceding claims 20 to 24, wherein the dedifferentiation step 1) comprises the treatment with 5-azacytidine for a

10 period of 18 hours.

26. The method according to the claim 24 or 25, wherein in the dedifferentiation step 1) the treatment is performed with 5-azacytidine 0.1 to 5 μ M.

15 **27.** The method according to the preceding claim, wherein in the dedifferentiation step 1) the treatment is performed with 5-azacytidine 1 μ M.

28. The method according to any one of the preceding claims, wherein the differentiation step 2) comprises

20 a three-step protocol, which includes the steps of:

2a) exposing the cells to activin A in pancreatic medium basal;

2b) exposing the cells to retinoic acid in pancreatic medium basal;

2c) exposing the cells to B27 supplement, basic fibroblast growth factor and insulin transferrin-selenium in pancreatic medium basal.

29. The method according to the preceding claim,
5 wherein step 2a) the cells are exposed to activin A having a concentration of 30 ng/ml.

30. The method according to the preceding claim,
wherein in step 2a) the exposure of cells to activin A is continued in step 2a) for a period of from 4 to
10 8 days and preferably of from 5 to 7 days.

31. The method according to the preceding claim,
wherein the exposure of cells to activin A is continued in step 2a) for a period of 6 days.

32. The method according to any one of the preceding
15 claims 25 to 28, wherein in step 2a) the medium is changed daily.

33. The method according to any one of the preceding claims 28 to 32, wherein in step 2b) retinoic acid 10 μ M is added to the cell medium.

20 **34.** The method according to the preceding claim,
wherein the treatment is performed for a period of from 2 to 3 days and preferably of 2 days.

35. The method according to any one of the preceding claims 28 to 34, wherein in step 2c) the medium
25 comprises 1% B27.

36. The method according to any one of the preceding claims 28 to 35, wherein in step 2c) the medium comprises 10 ng/ml basic fibroblast growth factor.

37. The method according to any one of the preceding
5 claims 28 to 36, wherein in step 2c) the medium comprises 1% insulin-transferrin-selenium.

38. The method according to any one of the preceding claims 28 to 37, wherein in the differentiation step 2) the cells are cultivated for at least 36 days.

10 **39.** The method according to any one of claims 21 to 31, wherein in step 2c) the medium is refreshed daily for the first 15 days, then it is refreshed every other day.

40. A cell, a population of cells, aggregates or
15 colonies or islets of cells obtained according to the method of any one of the preceding claims.

41. The cell, the population of cells, aggregates or colonies or islets of cells according to the preceding claim, which is capable of secreting
20 pancreatic hormones.

42. The cell, the population of cells, aggregates or colonies or islets of cells according to the preceding claim, wherein said hormone is selected in the group comprising insulin, C peptide,
25 somatostatin, glucagon.

43. A hormone produced by the cell, a population of cells, aggregates or colonies or islets of cells according to the claim 41 or 42.

44. An *in vitro* experimental model comprising the
5 cells, the population of cells, the aggregates of cells or the colonies of cell of any one of the preceding claims 40 to 42.

45. A method for treating pathologies related to the production or secretion of pancreatic hormones,
10 comprising the use of the cells, the population of cells, aggregates or colonies or islets of cells of any one of claims 40 to 42.

46. The method according to the preceding claim, wherein said pancreatic hormone is selected in the
15 group comprising insulin, C peptide, somatostatin, glucagon.

47. A method for controlling the level of pancreatic hormones, comprising the use of the cell, the population of cells, aggregates or colonies or islets
20 of cells of any one of claims 40 to 42.

48. A method for identifying the activity or toxicity of active principles capable of controlling the level of pancreatic hormone in a patient, comprising the use of the cell, the population of cells, aggregates

or colonies or islets of cells of any one of claims
40 to 42.

49. An implant comprising the cells, the population
of cells, aggregates or colonies or islets of cells
5 of any one of claims 40 to 42.

Fig. 1

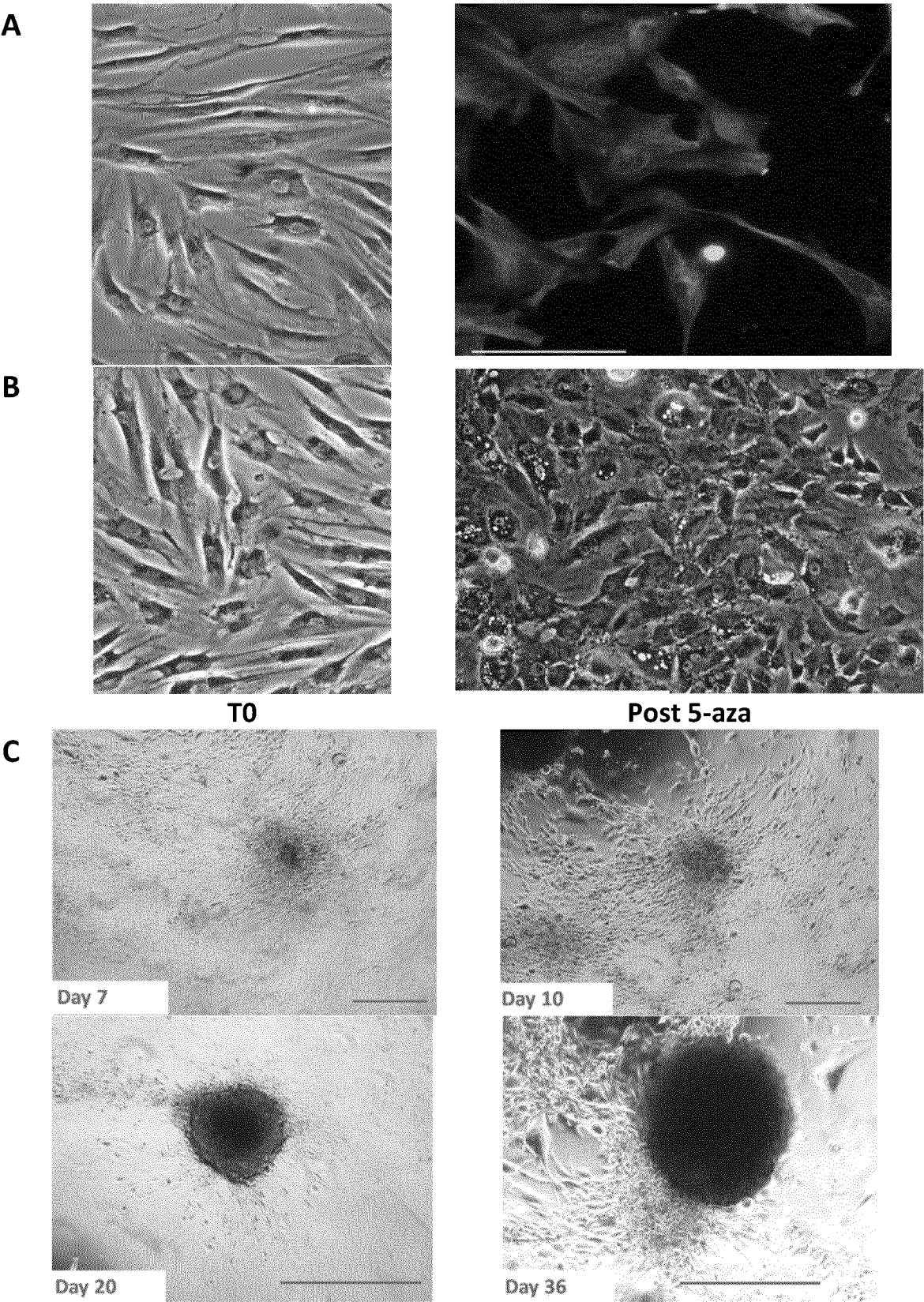


FIG. 2

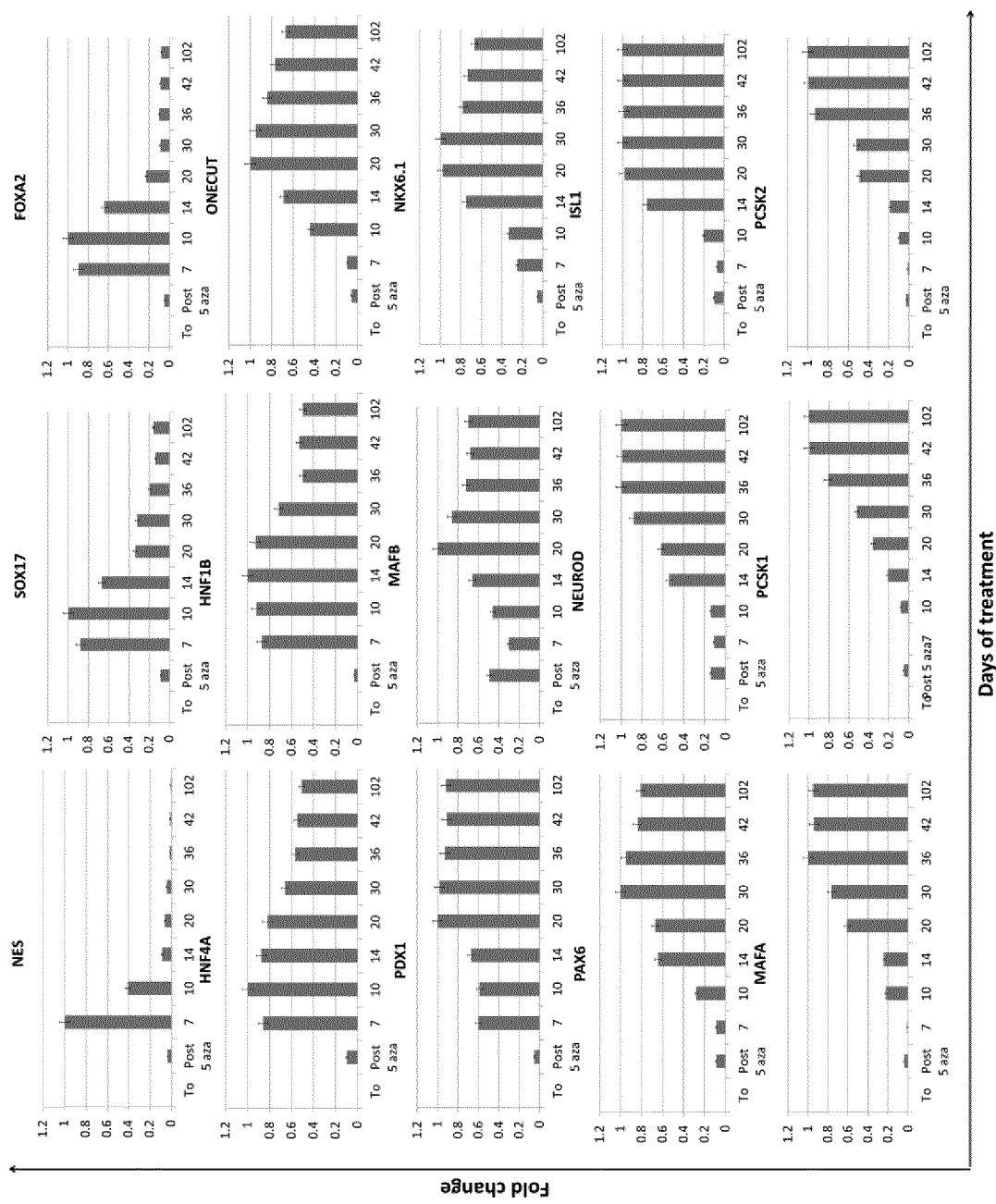
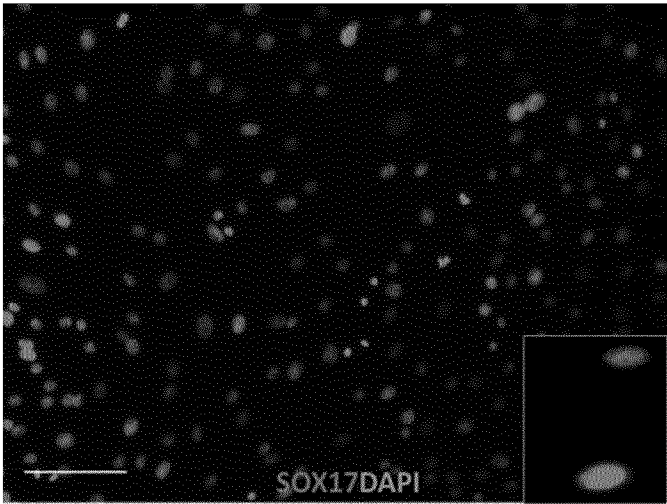


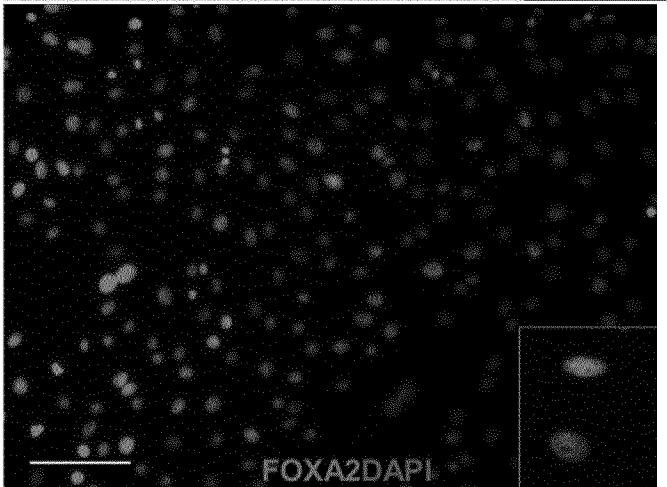
Fig. 3

A

SOX17DAPI



FOXA2DAPI



HNF4DAPI

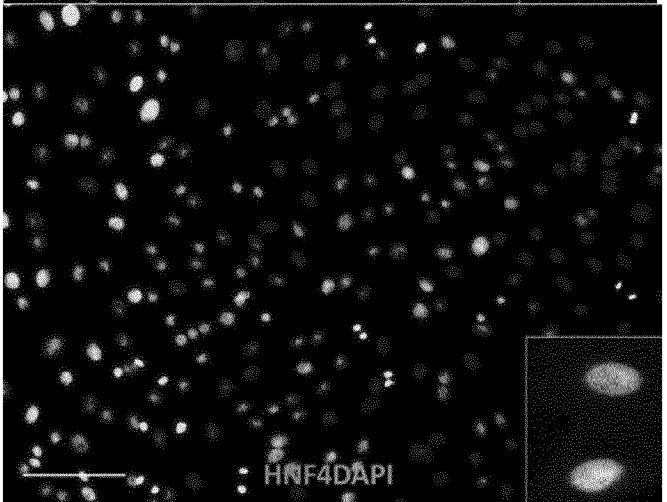
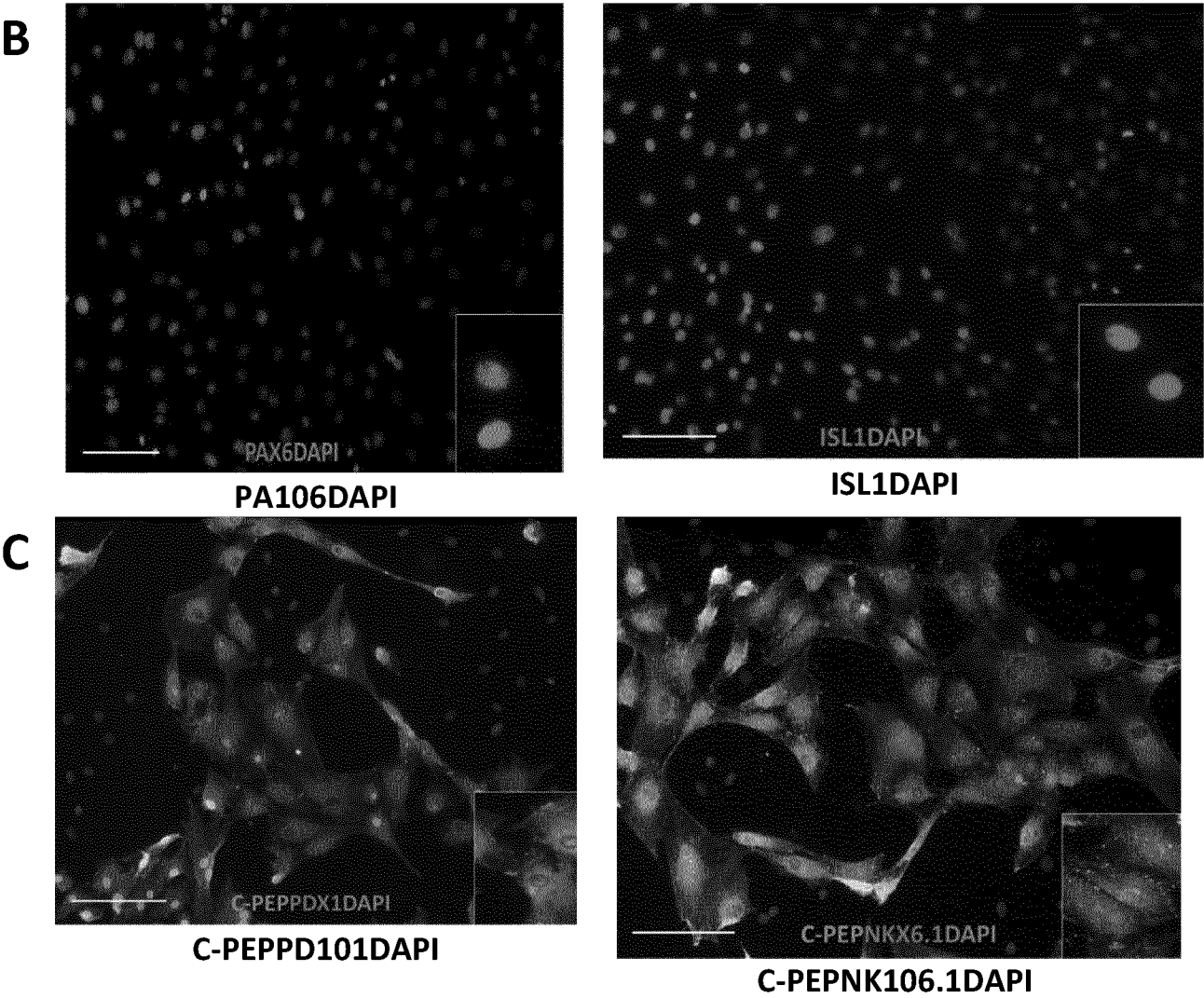


Fig. 3



	Days of treatment	PCC
SOX17	10	
FOXA2	10	
HNF4	20	
PDX1	20	
NKX6.1	30	
PAX6	30	
ISL1	30	

Fig. 3

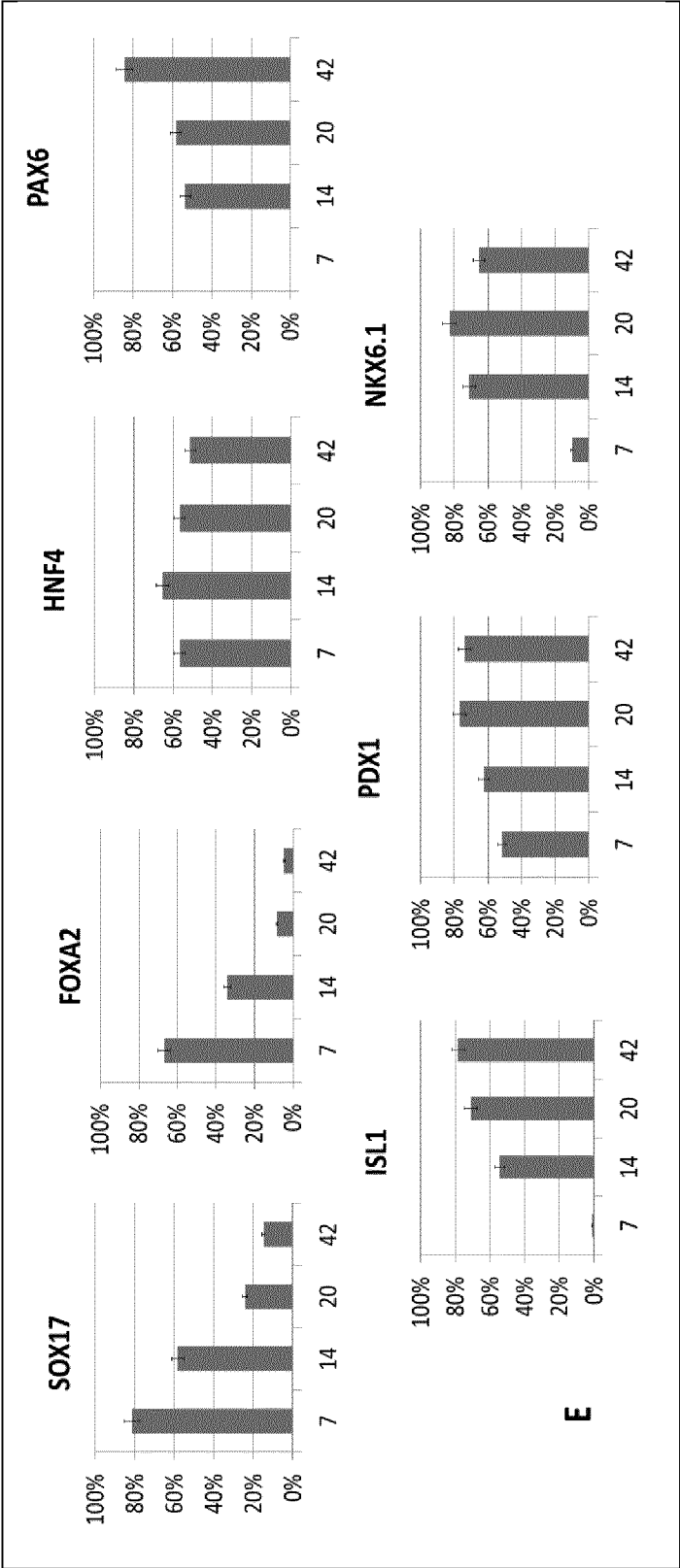
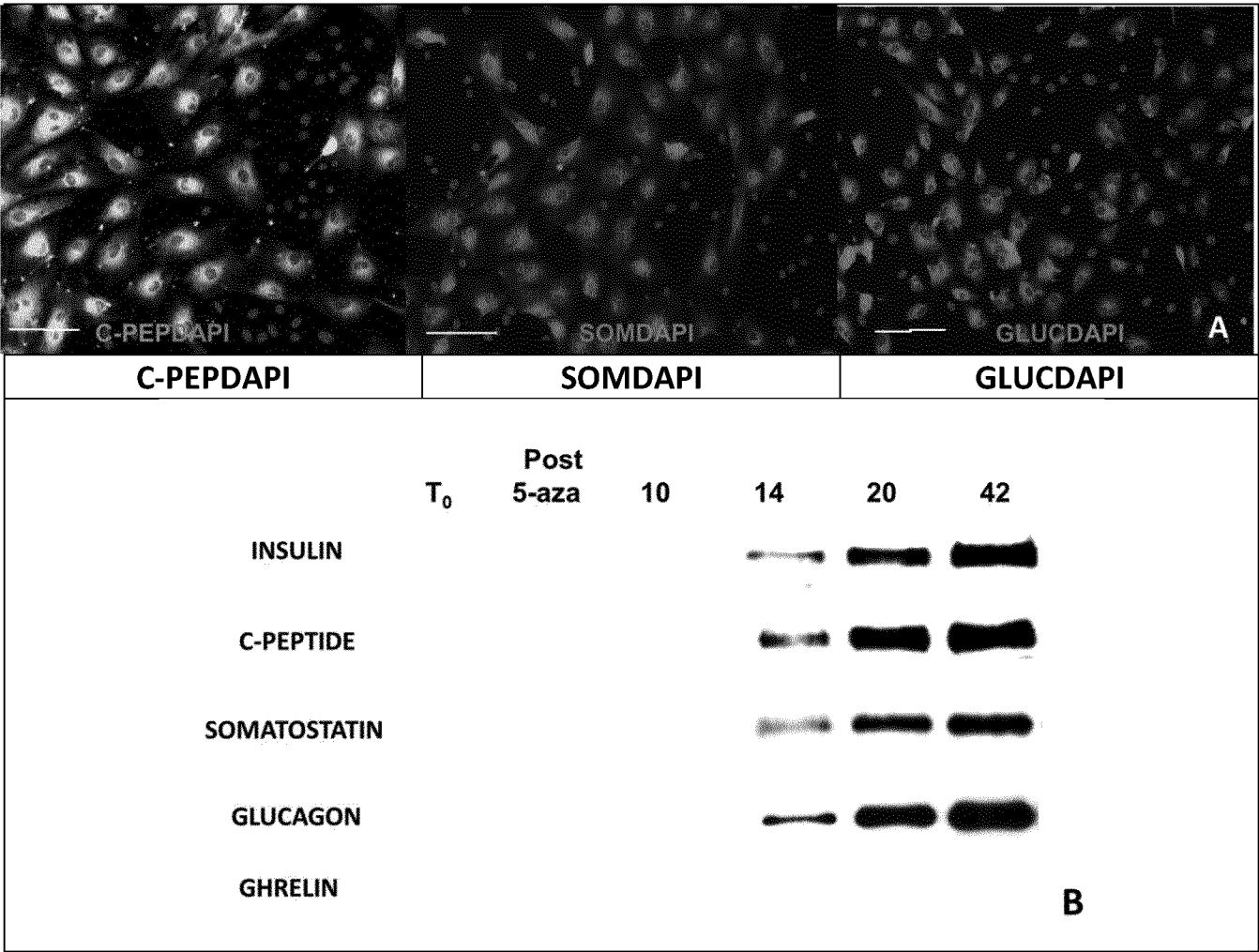


Fig. 4



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Fig. 4

C

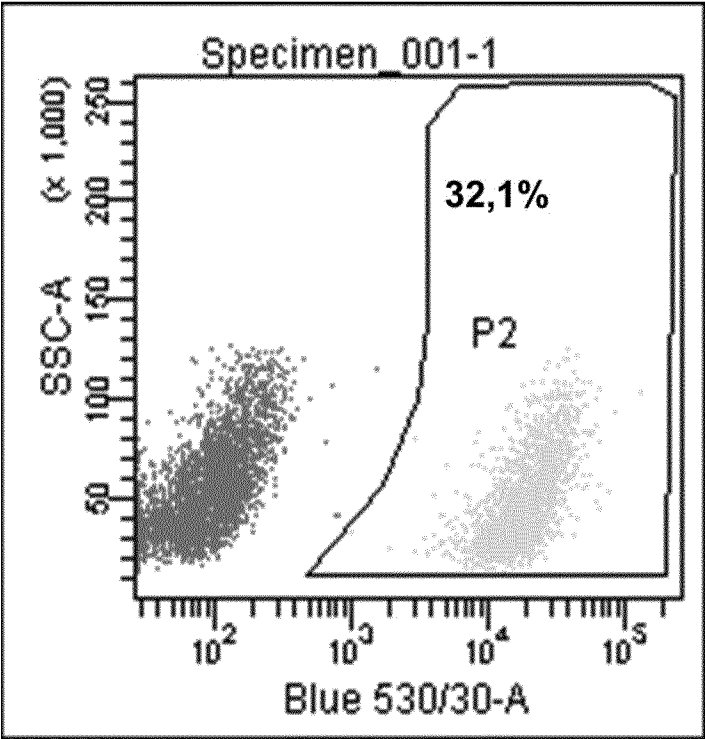
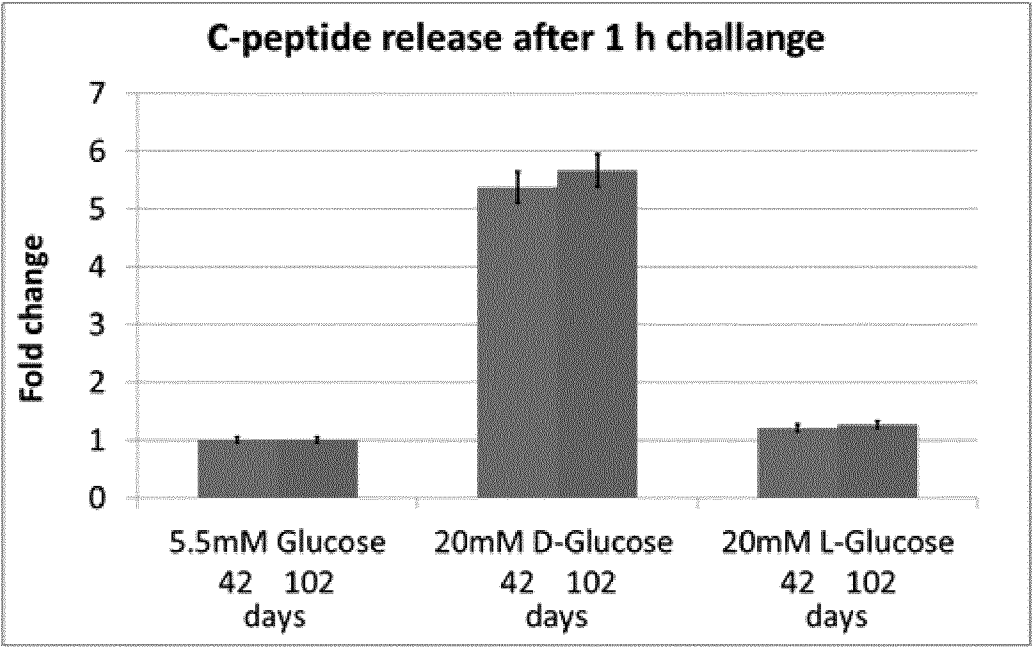


Fig. 4

D



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Fig. 5

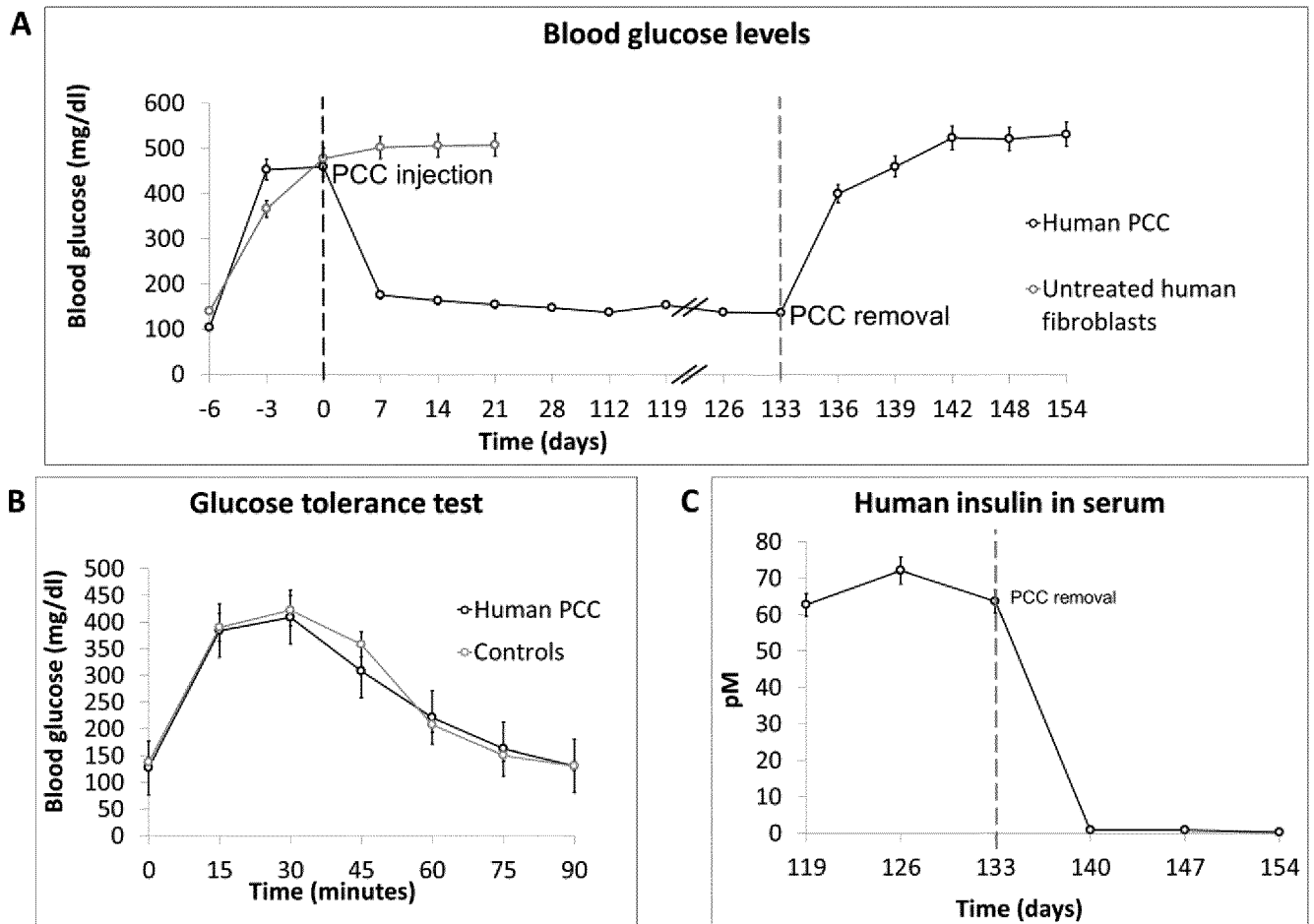
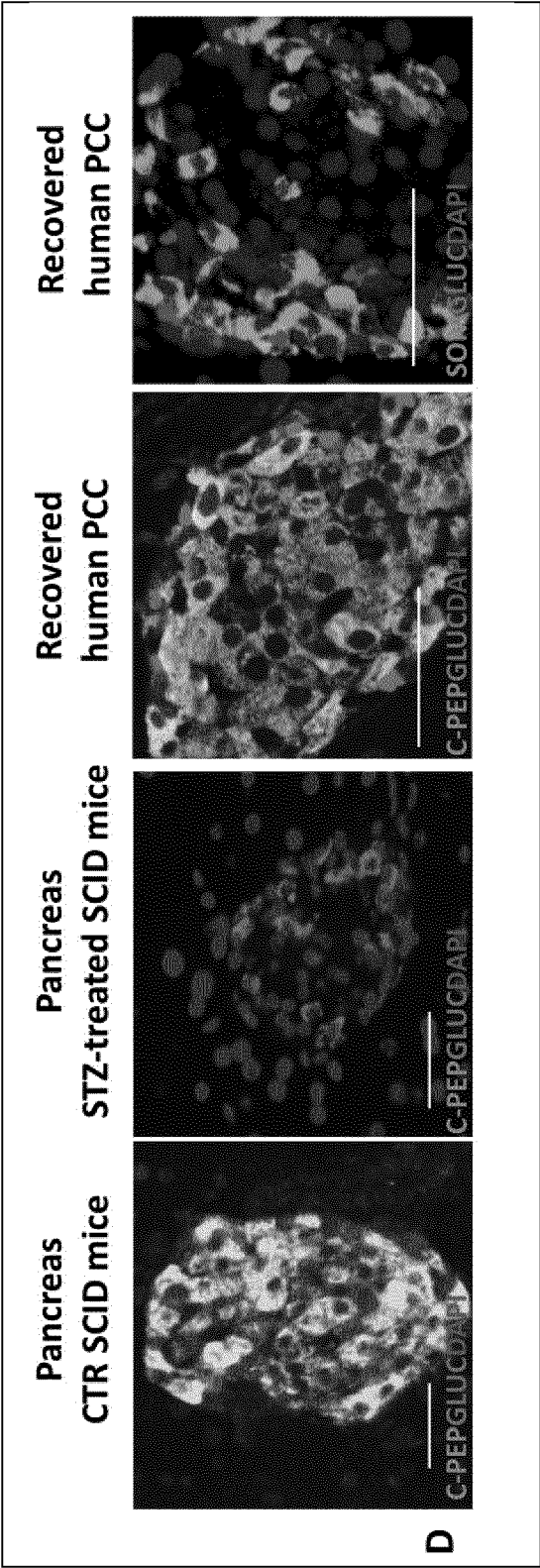


Fig. 5



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/058022

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N5/071 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, BIOSIS, Sequence Search, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "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 22 July 2014		Date of mailing of the international search report 29/07/2014
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Ferreira, Roger

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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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