Selection for CD49A+ and CD26- cells in pancreatic islet-like clusters differentiated from human pluripotent stem cells improves their therapeutic activity in diabetic mice

Kfir Molakandov (✉ k.molakandov@kadimastem.com)

Kadimastem <https://orcid.org/0000-0002-9749-3811>

Denise A. Berti

Kadimastem

Avital Beck

Kadimastem

Ofer Elhanani

Weizmann Institute of Science Department of Biomolecular Sciences

Michael D. Walker

Weizmann Institute of Science Department of Biomolecular Sciences

Yoav Soen

Weizmann Institute of Science Department of Biomolecular Sciences

Karina Yavriyants

Kadimastem Ltd.

Michal Zimerman

Kadimastem Ltd.

Ella Volman

Kadimstem Ltd.

Yitzhak Toledo

Kadimastem Ltd.

Anna Eruhimovich

Kadimstem Ltd.

Alon Levy

Kadimastem Ltd.

Arik Hasson

Kadimastem Ltd.

Joseph Itskovitz-Eldor

Kadimastem Ltd.

Judith Chebath

Kadimastem Ltd.

Michel Revel

Kadimastem Ltd.

Research

Keywords: Human ESC-derived insulin producing cells, Islet-like clusters (ILCs), Functional cell capture screening, MACS selection, Integrin alpha1, CD49A, alginate bead encapsulation, intraperitoneal implantation, STZ-treated C57Bl/6 mice diabetes models, diabetes cure

Posted Date: April 27th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-23915/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Cell therapy of diabetes aims at restoring the physiological control of blood glucose by transplantation of functional pancreatic islet cells. Human islets from post-mortem donations have shown efficiency but the demand for islets vastly exceeds the availability of donations. A potentially unlimited source of cells for such transplantations would be islet cells derived from in vitro differentiation of human pluripotent stem cells (hPSC), such as embryonic stem cells (hESC). The islet-like clusters (ILC) produced by the known differentiation protocols contain various cell populations. Among these, the beta cells that express both insulin and the transcription factor Nkx6.1 seem to be the most efficient to restore normoglycemia in diabetes animal models. Our aim was to find markers allowing selection of these efficient cells.

Methods

Functional Cell-Capture Screening (FCCS), using an array of antibodies to cell surface proteins, was used to identify markers that preferentially capture the cells expressing insulin, or expressing both insulin and Nkx6.1, from hESC-derived ILC cells. In order to test whether selection for such markers could improve cell therapy in diabetic mouse models, we used ILC produced from a clinical-grade line of hESC by a refined differentiation protocol adapted to up-scalable bioreactors. The ILC, dissociated to single cells, were fractionated by Magnetic Activated Cell Sorting (MACS) for presence of the marker. The sorted cells, re-aggregated into clusters, were encapsulated in microspheres made of alginate modified to reduce foreign body reaction. Implantation was done intraperitoneally in C57BL/6 immuno-competent mice that were made diabetic by prior injections of Streptozotocin (STZ).

Results

CD49A (integrin alpha1) was identified by FCCS as a marker for cells double positive (DP) for insulin (and C-peptide) as well as Nkx6.1 in ILC derived by hESC differentiation. After sorting by MACS with CD49A antibodies, the ILC fraction enriched in CD49A⁺ cells rapidly reduced glycemia when implanted in the diabetic mice, whereas mice receiving the CD49A depleted population remained highly diabetic. CD49A-enriched ILC cells also produced significantly higher levels of human C-peptide in mouse blood. Another marker, CD26 (DPP4, dipeptidyl peptidase-4), was identified by FCCS as binding insulin-expressing cells which are Nkx6.1-negative. Depletion of CD26⁺ cells followed by enrichment for CD49A⁺ cells increased DP cells to over 70%. After this double selection, the CD26 depleted/CD49A enriched ILC were more active than non-sorted ILC to reduce glycemia in the diabetic mice.

Conclusions

Refining the composition of ILC differentiated from hPSC by negative selection to remove cells expressing CD26 and positive selection for CD49A expressing cells can enable more effective cell therapy of diabetes.

Background

Diabetic conditions due to destruction (in type I) or dysfunction (in type II) of pancreatic islets of Langerhans, have detrimental impacts on the quality of life and lifespan. For the hundred million patients depending on frequent insulin injections for controlling blood glucose levels, islet cells transplantation would have the advantage to restore a physiological regulation of glycemia. The Edmonton protocol (1–3) based on transplantation through the portal vein of human islets obtained from brain dead donors has allowed patients to become insulin-free for significant periods of time. Yet, the availability of such islet donations is too limited to meet the transplantation demand. An alternative, more abundant supply may be generated by differentiation of islet-like clusters (ILC) from large-scale cultures of human pluripotent cells (hPSC). Recently developed multistage protocols of differentiation produce pancreatic islet cell populations including insulin-producing beta cells that function in reducing glycemia in animal models of diabetes (4–6). New methods for microencapsulation in modified alginate support long term function after implantation in diabetic animals (7–9).

Still, the differentiated ILCs contain additional populations of cells, which may not be necessary for islet function, and even may limit the efficacy and safety of the transplanted cells for future clinical treatment of diabetes. To alleviate this problem, we devised a method to identify and isolate the ILC cells that have higher capacity to normalize glycemia. This method is based on Functional Cell-Capture Screening (FCCS) on an array of antibodies, previously developed to identify cell-surface markers selective for endoderm and non-endoderm populations of differentiating hESC (10). The same procedure was later used to characterize surface markers of cells in post-mortem islets from adult humans (11). The present study was aimed to generate more potent ILC by applying FCCS to identify markers enabling selection of insulin-producing cells in cultures derived from a clinical-grade line of human ESC. We refined an appropriate 30-day protocol, in suspension cultures and larger bioreactors, which converts 3D clusters of these highly pluripotent stem cells into pancreatic islet-like clusters. By applying FCCS to these clusters dissociated into single cells, we identified several novel cell-surface markers which can be used to select cells expressing insulin and cells expressing both insulin and the Nkx6.1 transcription factor. Among these markers, CD49A (integrin- α 1) proved useful for positive selection of insulin-producing cells co-expressing NKX6.1. Further, CD26 (Dipeptidyl peptidase-4) was found selective for cells expressing insulin but not Nkx6.1, making it useful for negative selection to remove these cells and enrich more for insulin⁺/Nkx6.1⁺ cells. The potential of selecting for these markers to improve the therapeutic activity of hESC-derived ILC in diabetic mice was tested. Implantation of clusters after enrichment for the CD49A marker resulted in significantly higher levels of human insulin C-peptide in the mouse blood and maintained better normoglycemia than unsorted ILC. In contrast, depletion for the CD49A marker abolished the therapeutic effect and the implanted mice remained highly diabetic. Moreover, after depletion for the CD26 marker followed by enrichment for CD49A positive cells, the clusters were more active than non-sorted ILC to reduce glycemia. The identified markers show the feasibility of functional enrichment strategies to improve the therapeutic activity of hPSC-derived ILC in diabetes.

Methods

Human ES cell growth and differentiation to pancreatic beta-like cells

Highly pluripotent, clinical grade, human ES cells HADC-100 (12), (provided by Professor Benjamin Reubinoff, Hadassah Medical School, Jerusalem, Israel) were grown to confluent monolayers in essential E8 medium (Gibco, Cat#A1517001), with addition of penicillin and streptomycin (PS, Gibco, Cat#15140-122) on vitronectin-coated flasks (Gibco, Cat# A14700). Differentiation was performed on cell aggregates formed in spinner flasks during two days in dynamic suspension cultures. In brief, 48 hours before starting the differentiation protocol (day-2), non-differentiated cells were dissociated with Versene (Gibco, Cat#15040033). Single cells washed with PBS^{-/-} (Gibco, Cat#14190-094) were seeded in 500 ml disposable spinner flasks (Corning, Cat#CZ-3153), filled with 250 ml E8 medium containing 10 μ M Rock Inhibitor Y27632 (Cayman Chemical, #10005583-10), at concentration of $0.8-1 \times 10^6$ cells/ml. The spinner flasks were placed on an orbital shaker (Senova) at speed of 70 rpm in a humidified incubator set at 5% CO₂ and 37 °C. This resulted in the formation of ES cell clusters in suspension, as well as in cell proliferation. On day - 1, 80% of the E8 medium was replaced. On Day 0, the E8 medium was washed away (13) by letting the aggregates settle for 5 minutes and removing the supernatant with a pipette. Cells were washed with 250 ml PBS^{-/-}; after 3 min stirring in the incubator, PBS^{-/-} was replaced by 250 ml of stage1 differentiation medium. The media for the seven-stage differentiation protocol, refined on the basis of several published protocols (4, 5, 14–17), are detailed in additional files 1 and 2 (Table S1 and Table S2).

For Bioreactors, the hESC aggregation and differentiation was similarly done in the DASbox mini (Eppendorf) with online monitoring of culture parameters. Four parallel bottles containing 100–150 ml medium were seeded with $0.8-1 \times 10^6$ cells/ml 48 h before and washed with PBS just before differentiation as above. Medium changes were done batch-wise in the semi-closed system using peristaltic pumps. The differentiation protocol was the same as for spinner flasks.

Real time quantitative PCR

RNA was isolated from cells using the RNAeasy micro kit (Qiagen #74004) and purified from genomic DNA with RNase-free DNase kit (Qiagen #79254). The cDNA synthesis was done with the high capacity cDNA Reverse Transcription kit (Applied Biosystems 4368814). Transcript levels were measured by real-time qPCR using Taqman Fast advanced master mix (Applied Biosystems #4444557). The level of each gene was normalized to endogenous HPRT gene, using the $2^{-\Delta\Delta CT}$ method. The probes used for qPCR are listed in Table S3. The MARIS procedure (18) is described in Additional files.

Flow cytometry.

Samples from settled aggregates during or at the end of the differentiation process were dissociated with Accumax (Sigma, Cat# A7089) (0.35 ml per 300,000 estimated cells) for 8–10 minutes, after which the enzyme was blocked in 1 ml PBS^{-/-} with 10% FBS, and the cells were centrifuged (350xg, 3 min). For

external cell membrane labelling, cells were washed in PBS^{-/-}, and antibodies (e.g. anti-CD49A, as listed in supplementary Table S4) were directly added to the cell suspension in FACS buffer (0.5% BSA in PBS) followed by incubation at 4°C for 30 min. For internal antigen labelling (e.g. anti-human C-peptide and Nkx6.1, Table S4), cells after centrifugation were washed once in 1 ml PBS and fixed in 0.4 ml of 4% paraformaldehyde (PFA, EMS Cat# 15710), for 20 min at 4°C. After two washes with PBS cells were incubated for 1 h at 4°C in blocking solution (PBS with 5% Bovine serum albumin (BSA) and 3% horse serum) containing 0.4% Triton x-100 (Sigma, T6878), and washed once with PBS. The cell pellet suspended in 0.1 ml blocking solution (with 0.1% Triton x-100) containing antibodies, was incubated for 14–16 hours at 4°C, or for 1 h at room temperature (RT), and washed with PBS. When fluorescent tag-conjugated primary antibodies were used, fluorescence was read after this step in Flow Cytometer-BD FACS Canto II. Otherwise, cells were incubated in blocking solution containing 1:100 dilutions of the fluorescent tag-conjugated secondary antibody and washed with PBS before reading fluorescence.

Functional cell capture screening (FCCS) on antibody arrays.

Antibody arrays were printed in a Microgrid printer with solid pins (Total array Systems, BioRobotics, Cambridge, UK) on hydrogel-coated slides (Full Moon Biosystems, Sunnyvale, CA) using a panel of 235 monoclonal mouse anti-human antibodies (BD biosciences), each antibody being spotted at five different places in the array, as described before (10, 11) (US patent 2018 / 0369290 A1, Item 0081). The cell clusters were dissociated using TrypLE Express (Invitrogen Cat#12604) for 4 min, followed by quenching with 10% FBS in PBS, and centrifugation and resuspension in CMRL. The printed area of the array was blocked for 3 minutes with 1% BSA in PBS solution, before cell seeding, at about 0.5×10^6 cells/ml in 0.25–0.5 ml of CMRL medium, supplemented with 2 µl of DNase I (Ambion 2U/µl) and incubation was for 1 hour at 37°C. Excess cells were removed in a large volume of PBS and the arrays were fixed in 4% (w/v) paraformaldehyde for 10 minutes. Cells on the array were permeabilized in PBS, 0.2% (v/v) Triton x-100 for 20 min, washed twice with PBS and blocked for 45 min in blocking buffer (2% FBS, 2% BSA, 50 mM glycine in PBS). After blocking, arrays were washed twice with PBS and incubated for 2 hours at RT in blocking buffer with 0.1% of Triton x-100 containing the primary antibody guinea-pig anti-insulin (DAKO, A0564). Primary antibodies were removed, and arrays were washed three times with working buffer. Then, secondary antibodies were added in working buffer for 45 min at room temp: cy5 donkey anti-guinea–pig, (Jackson ImmunoResearch 706 – 175 -148). Arrays were washed three times in working buffer and imaged using automated, high content fluorescence microscopy (IXmicro, MDC). Total cells in each spot were counted by phase microscopy and the percent of insulin positive cells was calculated. Three repeats were performed with different batches of ES-derived cells at stage 7 of the differentiation protocol. The significance of the amount of cell binding to surface antibody was evaluated by two sample paired T-test (p value less than 0.05). In another set of experiments, cells were reacted with antibodies against insulin as above but also with antibodies against Pdx1 and against Nkx6.1 (see Table S4). The number of cells stained for PDX1 and Insulin, and for insulin and Nkx6.1 was counted.

Magnetic Activated Cell Sorting (MACS)

Cell clusters after stage 7 of differentiation were washed in PBS^{-/-}, dissociated with Accumax (10 ml for 25×10^6 cells) for 10 min at 37°C,, washed with CMRL 2% BSA and filtered through a 30 µm MACS filter previously washed with PBS^{-/-}, to eliminate cell aggregates, and counted using Nucleocounter® NC-200. Part of the clusters was set aside for control (non-dissociated, non-selected cells), seeded at 10^6 cells per ml in ultra-low binding 6-well-plates (Corning Cat#CLS3471) and left in the incubator on a stirrer (Dura Mag 9 position digital stirrer, CHEMCELL) set at 95 rpm in the 37°C incubator for three days in medium CMRL+ (Table 2s), before implantation. Cells are suspended in MACS buffer (PBS^{-/-}, 2% BSA, 2 mM EDTA, sterile, degassed (100 µl per 10^7 cells), for reaction with anti-CD49A (Myltenyi, cat# 130-101-397) using 10 µl per 10^7 cells for 10 min at 4°C, followed by washing with 5 ml MACS buffer. Cells suspended in MACS buffer (80 µl per 10^7 cells) were reacted with 20 µl per 10^7 cells of anti-PE microbeads (Milttenyi; cat# 5181214192) for 15 min at 4°C. After washing with 5 ml cold MACS buffer and centrifugation, cells were suspended in MACS buffer and applied to pre-separation filters and LS MACS column(s) as recommended by the manufacturer. Prior to implantation, all single cells fractions were re-aggregated in suspension in non-TC treated 6-well plates (Corning Cat#CLS3471-24EA) in CMRL +, 10 µM RI Y27632 and 2µ g/ml Laminin (Bio Lamina Cat#MX521CTG), on the stirrer, set at 95 rpm.

Table 2
Proportions of C-peptide and Nkx6.1 subpopulations in ILC cells double selected with antibodies to CD26 and to CD49A

Expt 1						
Cell fraction sorted	Total Non sorted	CD26 enriched	CD26 depleted	CD26 depleted CD49A enriched	CD26 depleted CD49A depleted	
Cell markers:	Percent of cells					
1. C-peptide/NKX6.1 DP	24.2	19.7	33.8	71.5		16
2. C-peptide only	22.9	43.0	4.8	10.7		4.0
3. NKX6.1 only	35.8	18.0	52.8	12.1		69.5
4. Negative	17.1	19.3	8.5	5.7		10.5
Expt 2						
Cell fraction sorted	Total	Total Reaggregated	CD26 enriched	CD26 depleted	CD26 depleted CD49A enriched	CD26 depleted CD49A depleted
Cell markers	Percent of cells					
1. C-peptide/NKX6.1 DP	22.5	32.6	6.1	33.7	66.6	21.2
2. C-peptide only	20.2	21.7	43.8	8.4	11.4	7.0
3. NKX6.1 only	13.3	21.6	6	24.3	13.3	42.7
4. Negative	44.1	24.2	44.1	33.6	8.7	29.1
The C-peptide/Nkx6.1 DP cells predominate in CD26 depleted/CD49 enriched fraction. Quantitation of double MACS sorting as described in Fig. 5. Two independent fractionation experiments are shown.						

For removal of CD26 positive cells prior to CD49A enrichment, the cells were dissociated as above, incubated for 10 minutes at 4°C with anti-CD26-PE (cat#302706), (10 ul/ 10⁷ cells, in 100 ul MACS buffer for 10⁷ cells), and after washing, reacted with anti PE antibody as described above. The mixture, after washing and resuspension in MACS buffer, was applied to LS column(s) and the flow through fraction (CD26 depleted) kept for further fractionation by MACS with anti-CD49A antibody.

Diabetes induction in mice and ILC implantation.

Immuno-competent mice C57BL/6J01aHsd (Harlan, Israel), 7-week-old males or female, were rendered diabetic by intraperitoneal injection of streptozotocin (STZ) (Sigma, Cat#S0130). As indicated, doses were either 150 mg STZ/kg, in one single injection, or 4 daily injections of 50 mg STZ/kg, after 6 hours fasting. Implantation was performed in diabetic mice, defined by blood glucose higher than 200 mg/dL for three consecutive days. Blood glucose was measured by a glucometer, on tail vein blood. Intraperitoneal Glucose Tolerance Test (IPGTT) was done after fasting the mice overnight by IP. injection of glucose (2 g/kg). Blood glucose was then monitored during a 2 hour period.

For implantation of micro-encapsulated ILC cells (see below), mice were anesthetized by an IP injection of ketamine/xylazine (Sigma, K4138) 85%/15% ratio (v/v) and then mounted on a surgical pad. The skin was prepared by shaving with electric clippers, application of Polydin, and then 70% ethanol solution. An abdominal incision (1 cm), and peritoneal incision (1 cm) allowed to insert microencapsulated ILCs into the peritoneal cavity of the mouse using a 1 ml sterile plastic tip (about 0.5 ml total volume). The peritoneum and the skin were closed with sutures and cleaned with Polydin. The mice were kept warm by a heating pad till they woke up. The cell doses implanted were between 1.0 and 2.5×10^6 cells, as indicated.

Preparation of TMTD-modified alginate and microcapsules.

Triazole thiomorpholine dioxide (TMTD Y1-Z15) preparation and coupling to alginate PRONOVA UP-MVG alginate (NovaMatrix) were done for Kadimastem, by Recipharm-Israel as described [8, 9]. After verification of the product structure by NMR, purification by filtration, dialysis and desiccation, elemental analysis revealed that more than 50% alginate guluronic or mannuronic residues were coupled to TMTD. Solutions of 4.6% of TMTD-coupled alginate were used (80% in volume of 5% (w/v) TMTD-coupled UP MVG and 20% of 3% (w/v) UP MVG). Stage 7 ILCs (see Table S1), washed with KREBS buffer without Ca^{++} pH 7.4, were mixed with alginate solutions at the concentration of about 10^7 cells /ml in a 5 ml Eppendorf tube. The micro-encapsulator Buchi B395, located in a tissue culture laminar flow hood for sterility, was set up to obtain microcapsules of 1.5 mm diameter with 4.6% alginate polymerized in CaCl_2 (100 mM in HEPES pH 7.4).

ILC Immunostaining and Imaging.

Stage 7 cell clusters fixed in 4% PFA and washed in $\text{PBS}^{-/-}$. A minimum volume of liquid 1% agarose was added to the pellet of clusters. After agarose became solid, the block was embedded in paraffin and 10 μm thick sections were produced and bound to glass slides. After removal of paraffin by alternative baths of xylene and ethanol, antigen retrieval was performed by heating slides in 10 mM citrate buffer pH 6.0 (ZYTOMED systems), by heating for 15 min in pressure cooker (Bio TintoRetriever). Blocking nonspecific binding and cell permeabilization was done by incubation with PBS containing 5% BSA, 3% horse serum and 0.3% Triton x-100 (blocking solution) 500 μl added per slide and slides covered with parafilm for 1 h at RT. Antibodies against PDX1, C-peptide, Nkx6.1 (Table S4) were diluted in blocking solution and incubation done overnight at 4°C in humidified chambers. After two washes in PBS, incubation with

secondary antibodies was done for 1 hr at room temperature and washes were done similarly. Nuclei were stained with DAPI (1 μ g/ml). The slides were mounted with aqueous mounting medium (2 drops) and covered with coverslip. Images were obtained using Nikon Eclipse 80.i fluorescence microscope and a DS.fi1 camera combined with NIS Elements computer program.

Results

Islet-like clusters differentiated from hESC contain mature beta-cells

Highly pluripotent hESCs were differentiated according to a 7-stage stepwise protocol carried out in suspension culture conditions (3D), in spinner flasks as well as in controlled bioreactors (as detailed in Methods). After stage 7 (day 30–34), the Islet-like clusters contain hormone-positive cells, especially cells producing insulin (as well as the C-peptide fragment processed from human proinsulin). These insulin-producing cells typically amount to 60% of the total cells, with about 10% of cells producing glucagon and 2% producing somatostatin (Fig. 1A,B). In addition to hormone-producing cells, the hESC-derived ILC still contain precursor cells, since over 90% of cells express the key transcription factor (TF) for pancreatic development PDX1 and 70–80% express TFs important for beta cell function such as Nkx6.1 and NeuroD1 (Fig. 1B). Nkx6.1 is of particular importance, being essential for development and function of mature beta cells (19, 20), and serving as a marker of mono-hormonal insulin-producing beta cells (21). In the hESC-derived ILC, these beta cells can be identified by flow cytometry (FACS) as double positive (DP) for Nkx6.1 and human C-peptide (Fig. 1C). This C-peptide⁺/Nkx6.1⁺ DP fraction usually represents around 20–40% of the total population, the rest being C-peptide⁺/Nkx6.1[−] (C-pep only), C-peptide[−]/Nkx6.1⁺ cells (Nkx6.1 only) and C-peptide[−]/Nkx6.1[−] (Negative, Q3) cells (Fig. 1C). The four subpopulations were characterized by gene expression. After separation by preparative FACS, RNA from each of the fixed and stained cell fractions was extracted and analyzed following the MARIS method (18). The qPCR data relative to the unsorted cells (Fig. 1D) confirmed that the C-peptide⁺/Nkx6.1⁺ DP compartment is enriched for cells that primarily express insulin. On the other hand, the C-peptide-only fraction is enriched for cells expressing Insulin but also Glucagon (GCG), Somatostatin (SST) and Pancreatic Polypeptide (PPY), identifying these cells with the reported poly-hormonal precursors (22, 23). Among the four sorted cell fractions, the C-peptide⁺/Nkx6.1⁺ DP cells had, in addition to insulin, the highest expression level of the transcription factors MafA and Nkx6.1, of the prohormone convertase PCSK1 and the GLP1 receptor (GLP1R) (Fig. 1D). These are characteristics of more mature beta cells. The Nkx6.1-only fraction showed also enrichment for PCSK1, GLP1R and Nkx6.1 expression, but had lower MafA and very low insulin, suggesting that these are pre-hormonal progenitors. The negative fraction contained cells expressing GCG, SST and PPY (Fig. 1D), suggesting the presence of maturing alpha, delta and epsilon islet cells respectively.

Table 1

Proportions of C-peptide and Nkx6.1 subpopulations in ILC cells selected for binding to CD49A antibodies

		Expt 1		Expt 2	
Cell Fraction:	Total	CD49A	CD49A	CD49A	CD49A
	Non sorted	enriched	depleted	enriched	depleted
Cells positive for markers:	Percent of cells				
1. C-peptide / Nkx6.1 (DP)	17.8	46.2	13.3	44.2	16.2
2. C-peptide only	24.8	19.7	22.2	28.6	26.8
3. NKX6.1 only	9.2	10.7	22.8	21.4	50.4
4. CD49A (overall)	23.8	61.4	9.5	52.8	10
5. C-peptide / Nkx6.1 / CD49A	ND	40.1	5.0	35.9	2.9
The C-peptide/Nkx6.1 double positive population is predominant in the cells binding to CD49A antibodies (CD49A-enriched). Quantitation of cells non-sorted or sorted with anti CD49a antibody by MACS and further reacted with anti-human C-peptide, anti-Nx6.1, and anti-CD49 antibodies. Cells from two independent batches were analyzed in two different experiments.					

Identification of CD49A as a marker for mature beta-cell

We used the FCCS platform (10) for identifying antibodies to cell surface proteins that preferentially capture insulin-producing cells (Fig. 2A). Single cells from dissociated ILC were incubated on the array and antibody-bound cells were then stained for insulin. Out of the 235 antibodies in the array, 61 of them captured some ILC cells. Of these antibodies, anti-CD49A consistently captured insulin⁺ cells in amounts exceeding 33% of the total cells captured (the median value for all antibodies being 13%). As illustrated in Fig. 2A, there were other antibodies binding insulin⁺ cells (e.g. CD99) and several that captured almost only insulin-negative cells (e.g. CD66C, CD73). These latter antibodies may serve for negative selection to remove cells that do not express insulin.

As a marker for positive selection of beta-cells, we chose CD49A (Integrin alpha-1) since further experiments showed that this marker is predominantly present in the C-peptide⁺/Nkx6.1⁺ DP subpopulation (Fig. 2B, blue). Magnetic activated cell sorting (MACS) with antibodies to CD49A, was used to fractionate live ILC cells into CD49A enriched and CD49A depleted populations. A marked increase in the percentage of C-peptide⁺/Nkx6.1⁺ DP cells was observed by FACS in the CD49A enriched fraction compared to CD49A depleted fraction or non-sorted ILC cells (Table 1, line 1), while there was no enrichment of C-peptide-only cells (line 2). Thus, within the CD49A-enriched fraction, the C-peptide⁺/Nkx6.1⁺ DP cells became relatively more abundant than the C-peptide-only and NKX6.1-only cells. In contrast, in the CD49A depleted fraction, the Nkx6.1-only cells were predominant (Table 1, line 3). While the proportion of CD49A⁺ cells increased to about 50–60% in the enriched fraction, there were still around 10% of CD49A⁺ cells in the depleted fraction (Table 1, line 4). This indicates that even after two

passages on a MACS column with anti-CD49A, the separation was not complete, explaining why there remained C-peptide-only and Nkx6.1-only cells in the CD49A enriched fraction. Yet, there may be heterogeneity in the distribution of CD49A. For example, when the CD49A-enriched fraction was triple stained for CD49A, C-peptide and Nkx6.1, it was found that 81–86% of the C-peptide⁺/Nkx6.1⁺ DP cells were positive for CD49A (compare lines 5 to line 1 in Table 1). However, the same comparison shows that most of the DP cells remaining in the CD49A-depleted fraction did not express CD49A (only 18–38% of them scoring positive for CD49A). This suggests that the C-peptide⁺/Nkx6.1⁺ DP population may be heterogeneous, some with and some without CD49A.

The CD49A-enriched MACS fraction had significant differences in gene expression as compared to the CD49A depleted fraction (Fig. 2C). After enrichment for CD49A⁺ cells (confirmed by the increase in ITGA1 transcripts encoding CD49A), there was an increase in mRNA for insulin, urocortin-3 (UCN3) and most significantly for MafA. The level of glucagon RNA was decreased but, unexpectedly, somatostatin RNA was slightly increased, suggesting enrichment of the relatively small population of delta cells. Interestingly, transcripts of a hepatic lineage gene (AFP), of which low amounts still remain in the hESC-derived ILC, are further decreased in the CD49A enriched MACS fraction (Fig. 2C). In addition, TPH1, a gene of the serotonin synthesis pathway, was also reduced (Fig. 2C), suggesting that CD49A enrichment removes non-beta cells producing serotonin inhibiting insulin secretion (24). Overall, the gene expression data confirm that selection for the CD49A cell surface antigen helps to enrich for functional mature beta cells.

In order to be able to transplant cells in large enough amounts, the selection method needs to perform in large-scale preparations. Cells dissociated from ILC at day 35 of differentiation (200 million cells), were fractionated by two consecutive passage on MACS columns with anti-CD49A. The twice retained fraction contained 75 million cells. The quality of this CD49A enriched preparation, evaluated by qPCR (Table S5), was similar to that of small-scale preparations, with increased Insulin, MafA and UCN3 mRNA, decreased GCG and IAPP mRNAs, relative to non-sorted and CD49A depleted cells. Thus, selection for the CD49A marker is effective to prepare the amounts of ILC needed for implantation experiments.

CD49A selection separates ILC cells that normalize glycemia in diabetic mice from inactive cells.

Based on the above in vitro evidence for enrichment of beta-cells, we proceeded to test the cells obtained by selection for the CD49A marker in a mouse diabetes model. Current methods for implantation of human ILC are based on micro-encapsulation in alginate spheres, so as to reduce direct contact of the cells with host immune cells. While the introduction of alginate spheres into the peritoneal cavity of immunocompetent mice has been shown to elicit foreign body reaction (FBR) and fibrosis even without ILC (8, 9), this reaction can be inhibited by using chemically modified alginate. In particular, long-term functionality was demonstrated for ILC transplants that were encapsulated in low-viscosity SLG20 alginate carrying triazole-thiomorpholine dioxide (TMTD-alginate) and implanted intraperitoneally (IP) in mice with streptozotocin (STZ)-induced diabetes (9).

In the present study, we used an alginate of medium viscosity (UP MVG alginate) coupled with TMTD for ILC microencapsulation. To establish a relevant baseline, we began by characterizing the glycemic normalizing capacity of ILC that were not refined by prior sorting. Spheres containing a total of 2.2 million cells (1,800 clusters) were injected IP. into female C57BL/6 mice that received 4 prior injections of low dose STZ (50 mg/kg). The mice had marked hyperglycemia for two weeks before implantation and showed a rapid decrease of blood sugar following implantation, reaching within 6 days the normoglycemic range (Fig. 3A). The average blood glucose remained in the normal range (below 200 mg/dl) over a follow-up period of 100 days (Fig. 3A). Non implanted mice remained highly hyperglycemic and died around day 40 of this follow-up period. In the implanted animals, we also examined the correlation of glycemia with human C-peptide levels in the blood. From two representative mice, it can be seen (Fig. 3B) that the one with higher C-peptide levels (broken red line) maintained lower glycemia (solid red line) whereas the one with lower C-peptide (broken blue line) had more incidences of mild hyperglycemia (solid blue line).

The efficiency of CD49A-enriched and depleted ILC cells was compared by measures of blood C-peptide levels and effects on glycemia. The ILC were dissociated into single cells, fractionated on MACS columns and then re-aggregated into clusters before micro-encapsulation in TMTD-alginate and implantation at a dose of 1.9 million cells. The CD49A-enriched ILC produced significantly higher levels of C-peptide than implants of ILC that were not dissociated nor sorted (Fig. 4A). Compared to fasting mice (blue bars), the C-peptide secretion was stimulated 30 minutes after glucose injection (red bars), demonstrating typical beta-cells response to glucose. The CD49A depleted ILC, which lack most of the C-peptide⁺/Nkx6.1⁺ DP population (Fig. 4B, right panel), had much reduced levels of basal and glucose-stimulated C-peptide levels (Fig. 4A). Accordingly, in the diabetes model, the CD49A-depleted ILC were unable to decrease glycemia and the mice remained highly diabetic (Fig. 4C, triangles) like mice without implant (dotted line). In contrast, the CD49A-enriched MACS fraction rapidly reduced glycemia (Fig. 4C, squares), which remained at relatively low levels over the follow-up period of 11 weeks. Although there were some events of mild hyperglycemia from day 20 to 45 (Fig. 4C), the prolonged activity of the CD49A-enriched ILC was attested by the fact that blood C-peptide levels of 155 pM were still observed on day 61, while none was found in mice that had received CD49A depleted cells (not shown). These data establish that selection for the CD49A surface marker separates the ILC cells that control glycemia from inactive ones. On the other hand, the data show a clear difference between the CD49A-enriched ILC and non-sorted ILC only after day 30 (Fig. 4C, red circles). While maintaining low glycemia during the first weeks, the non-sorted ILC lost their ability to do so, leading to death before day 45 (Fig. 4C). The more limited effect of the unsorted ILC (as compared to the experiment in Fig. 3A) is likely due to the lower cell dose implanted.

Combining negative selection for CD26 with positive selection for CD49A

Selection for the CD49A marker increases the percentage of C-peptide⁺/Nkx6.1⁺ DP cells but does not remove the C-peptide-only fraction (Fig. 4B left panel, Table 1). To improve the selection, we attempted to identify surface markers of ILC cells expressing Insulin and Nkx6.1 together (DP cells). In a new FCCS, each spot of the antibody array was evaluated for capture of insulin⁺-cells and then for the percentage of

Nkx6.1⁺ cells among them. We were unable to find an antibody that captured specifically DP cells, but observed that an antibody to CD26 (Dipeptidyl peptidase-4, DPP4), which captured efficiently insulin⁺ cells (70% of all the cells bound), failed to capture DP cells (3.5% DP among all insulin⁺ cells, Table S6). This offered the possibility to remove insulin-producing cells that lacked Nkx6.1. MACS selection with anti-CD26 showed that in the unbound cell fraction (CD26 depleted) the C-peptide⁺/Nkx6.1⁻ cells were markedly reduced whereas the DP cells and the Nkx6.1-only cells were increased (Fig. 5, Table 2). By a subsequent selection for the CD49A marker, the percentage of DP cells was raised to 70% in the CD26 depleted and CD49A enriched fraction (Table 2, lines 1).

To evaluate their activity for diabetes therapy, implants of CD26 depleted/CD49A enriched ILC were compared to total non-sorted ILC. We observed that even when the dose of cells implanted was reduced to 1 million cells per mouse, the CD26 depleted/CD49A enriched ILC still showed efficiency to rapidly reduce glycemia and maintain it in the normal level (Fig. 6A, green squares). Under these conditions, the non-sorted ILC did not bring down glycemia to normal levels, but only reduced it slightly and maintained it at an intermediate diabetic state (Fig. 6A red circles). The CD49A depleted fraction obtained from the CD26 depleted cells, did not maintain the glycemia, which rose to highly diabetic levels (Fig. 6A, triangles).

The levels of human C-peptide in the blood of mice implanted with CD26 depleted/CD49A enriched ILC were higher than with the other types of ILC, throughout the follow-up period of 50 days (Fig. 6B). This was observed in fasting mice and after stimulation of C-peptide secretion by glucose. As compared to non-sorted ILC, the increase in C-peptide secretion after combined CD26 and CD49A selection was more pronounced than that observed after CD49A selection alone (Fig. 6B versus 4A). In correlation, the cell fraction selected by CD26 depletion and CD49A enrichment was smaller than after CD49A-enrichment alone (respectively 25% versus 38% in a representative comparison). Thus, the double selection appears as a promising strategy to improve cell therapy of diabetes with hPSC-derived ILC.

Discussion

We show here that it is possible to enrich for a cell population that is active to reduce glycemia in diabetic mice by fractionation of the cell populations produced by differentiation of human pluripotent stem cells towards pancreatic endocrine lineages. To find markers for selection, we used a functional cell capture screening (FCCS) assay on a microarray of large set of antibodies to cell surface proteins (10).

In a first series of screens, we identified an antibody to integrin-alpha1 (CD49A, ITGA1) as efficiently binding insulin-producing cells. We show that by binding to anti-CD49A, one can purify cells that reduce glycemia upon implantation to diabetic mice and separate them from cells lacking this capacity. In a second series of screens, in which capture of insulin-producing cells was compared to that of Nkx6.1⁺ cells, we found that an antibody to dipeptidyl peptidase-4 (CD26, PPD4) bound specifically ILC cells expressing insulin (human C-peptide) but lacking Nkx6.1. By negative selection to remove CD26⁺ cells followed by a positive selection enriching for CD49A⁺ cells, a cell population containing over 70% C-

peptide⁺/Nkx6.1⁺ DP cells could be obtained. This purified ILC fraction had an improved efficacy to reduce glycemia relative to non-sorted ILC, and less cells were needed for therapy of diabetic mice.

Recent studies (25, 26) used single-cell RNA Seq analysis to study gene expression in the various cell populations present at the end of in vitro differentiation protocols producing islet cells. Through this approach, Veres et al (26) found that CD49A (ITGA1) is a marker of mature beta cells expressing the INS, Nkx6.1, NPTX2, PCSK1 genes, and showed that MACS with CD49A antibodies can isolate these beta cells at 60–80% purity. These authors reported that, in vitro, glucose-stimulated insulin secretion (GSIS) was improved in their CD49A⁺ MACS-purified fraction. Our approach was different, and we identified the CD49A marker by screening of antibodies against proteins of the plasma membrane. Our data further demonstrate that only the ILC cells binding to CD49A antibodies are active to normalize glycemia in vivo, whereas the unbound cells are unable to do so. The CD26 depleted/CD49A enriched cells give better therapeutic activity in the mouse diabetes model and show improved GSIS in vitro (not shown).

The FCCS assay has been previously applied to cells of natural pancreatic islets from human post-mortem donations and identified the combination of CD56 and CD9 antibodies as able to select insulin-producing beta cells (10). With our hESC-derived ILCs, CD56 was also high on the list of antibodies binding insulin-expressing cells in the FCCS assay, while CD9 was not found (not shown). The CD200 marker that had been reported to isolate endocrine cells present among pancreatic progenitors differentiated from hESC (22), was also found in our FCCS (not shown).

Regarding the role of CD49A, it is known that this Integrin alpha1 chain has a function in the development of beta cells in the human fetal and adult pancreas since, in combination with Integrin-beta1, it forms the primary collagen-binding receptor (in particular for Col IV) and thereby contributes to beta cell adhesion and motility, as well as insulin secretion (27). Such effect of Col IV on Insulin secretion could possibly be exploited to further enhance biological activity in the selected CD49A⁺ cells. Moreover, the presence of CD49A may be important for the function of beta cells in vivo. This could explain why the CD49A-depleted ILC fraction has no effect on diabetes: even though this fraction still contains some DP cells, the CD49A protein is absent in most of them (Table 1, compare line 5 to line 1).

The MACS purification requires dissociation of the islet-like clusters that are produced in our 3-dimensional differentiation process. We chose to re-aggregate the cells into clusters prior to their implantation to mice, by adding a few days of culture after the MACS fractionation. This procedure does not alter the composition of the ILC, nor their activity in diabetes models (28). Therefore, we compared the selected ILC to either intact ILC or re-aggregated non-sorted ILC in different experiments. In the present work, we used medium viscosity MVG alginate coupled with TMTD to microencapsulate the clusters, and not the very low viscosity SLG20 alginate as used by Vegas et al (9). The TMTD-MVG microspheres (of 1.5 mm diameter) implanted in the peritoneal cavity of immunocompetent C57BL/6 mice, made diabetic by prior STZ treatment, caused a rapid decrease in glycemia starting immediately after implantation. This is an indication that the in vitro differentiated ILC provide the functionally mature beta and other cell types needed to restore and maintain glycemia in the normal range. The same was observed with the ILC

cells fractionated by binding to the marker antibodies. Yet, considering that the MACS fractionation is not complete, the enriched fractions may still include other cell types that influence the therapeutic effect. Indeed, it is likely that the presence of glucagon-producing alpha cells is important to prevent hypoglycemic events. Our results show that the cells which do not bind to the CD49A antibodies are unable to reduce glycemia and the mice receiving these cells behaved as the non-implanted controls. Cells of this CD49A-depleted MACS fraction still express some insulin, but also have high glucagon gene expression (Fig. 2C, Table 5s). This was also the case for the CD26-depleted/CD49A-depleted cells (not shown). The CD49A depleted cells contain the C-peptide-positive/Nkx6.1-negative (C-peptide-only) cell population seen by flow cytometry (Fig. 4B), which are polyhormonal progenitors that do not contribute beta cell functions, and can become alpha cells in vivo (22, 29). These C-peptide-only cells are eliminated in the CD26-depleted/CD49A-enriched fraction (Fig. 5). The same applies to Nkx6.1 positive cells lacking C-peptide (likely to be progenitors) and cells lacking both C-peptide and Nkx6.1 (containing also some non-beta islet cells). The CD49A-enrichment also removes cells expressing TPH1 (Fig. 2C), which probably correspond to cells producing serotonin (entero-chromaffin cells) that inhibit insulin secretion (24,26). Removing these populations not only eliminates cells that do not participate in the control of glycemia or even disturb this control, but helps reducing the number of in vitro differentiated cells that need to be implanted for cell therapy of diabetes. The double selection, depleting CD26⁺ cells and enriching for CD49A⁺ cells, removes many unwanted cells, and it indeed allowed to reduce the number of cells needed for normalizing glycemia. This reduction could be of practical importance for therapy, as it would decrease the volume of hPSC-derived ILCs that will be required to treat human diabetic patients.

The FCCS assay identified additional antibodies that may be candidates to select cells active in restoring and maintaining normoglycemia in diabetics, and/or remove inactive cells by negative sorting (data not shown). Development of new means of large-scale cell sorting with anti-CD49A and other combinations of antibodies appears as an important endeavor for treating the many millions of diabetic patients by regenerative cell therapy.

Conclusions

In vitro differentiation of islet-like clusters from human pluripotent stem cells represents a potentially unlimited source of cells that could restore physiological control of blood glucose in diabetic patients requiring insulin. The CD49A surface protein (integrin- α 1) was identified as a selective marker of the ILC cells that are most active to normalize glycemia in a diabetic mice model. Removal of cells expressing CD26 (DPP4) prior to enrichment of the cells binding to CD49A antibodies further reduced the number of ILC cells needed to normalize glycemia.

Abbreviations

AFP: alpha fetoprotein; BSA: bovine serum albumin ; CMRL : CMRL serum free medium; Col: collagen; DP: double positive; DPP4: dipeptidyl peptidase-4; ES: embryonic stem; FACS: fluorescent activated cell sorting; FBS: fetal bovine serum; FCCS: functional cell capture screening; GCG: glucagon; GLP1R:

glucagon-like peptide-1 receptor; GSIS: glucose stimulated insulin secretion; HPRT: hypoxanthine phosphoribosyl transferase; hPSC: human pluripotent stem cell; hESC: human embryonic stem cell; IAPP: islet amyloid polypeptide; ILC: islet-like cluster; IP: intraperitoneal; IPGTT: intraperitoneal glucose tolerance test; ITGA1: integrin alpha1; MACS: magnetic activated cell sorting; MafA: Pancreatic Beta-Cell-Specific Transcriptional Activator; Nkx6.1: Nk6 homeobox-1; NMR: nuclear magnetic resonance; NPTX2: neuronal pentraxin-2; PBS-/ -: phosphate buffered saline, no calcium, no magnesium; PCSK1: prohormone convertase; PFA: paraformaldehyde; PPY: pancreatic polypeptide Y; qPCR: quantitative polymerase chain reaction; RT: room temperature; SST: somatostatin; STZ: streptozotocin; TF: transcription factor; TMTD: triazole-thiomorpholine-dioxide; TPH1: tryptophan hydroxylase-1; UCN3: urocortin-3.

Declarations

Ethics approval

This study was performed following an application-form review by the National Council for Animal Experimentation and after receiving approval (No. IL-19-7-313) that the study complies with the rules and regulations set forth.

Consent to participate: not applicable

Consent for publication: not applicable

Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

Authors KM, DB, AB, KY, MZ, IT, AE, AL, AH, JI, JC, are researchers employed in the Biotechnology Company Kadimastem Ltd, 7 Sapir Street, Nes Ziona, Israel. MR is a major shareholder of Kadimastem.

Funding:

This work was supported by the Israel Innovation Authority grants No. 51743, 54659 and 56956

Author's contributions:

KM, JC and MR directed the research work and wrote the manuscript. OE, MW and YS conceived and contributed the Functional Cell Capture Screening. DB, AB, KY, MZ, IT, AE performed the experiments. AL, KM supervised the animal experimentation. AH, JI were senior advisors. All authors read and approved the final manuscript.

Acknowledgements

We acknowledge with thanks the valuable contribution of Dr Raanan Margalit, CEO, Science In Action company, where all animal experimentations were performed.

Author's information

Dr Denise Berti present address: Senior Staff Scientist, Maiké Sander Lab, Dpt of Pediatrics, School of Medicine, University California, San Diego, CA, USA.

References

1. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. *Nat Rev Endocrinol*. 2017;13(5):268–77.
2. Gamble A, Pepper AR, Bruni A, Shapiro AMJ. The journey of islet cell transplantation and future development. *Islets*. 2018;10(2):80–94.
3. Shapiro AMJ. Islet Transplantation - The Canadian Perspective. *CellR4 Repair Replace Regen Reprogram*. 2019;7.
4. Pagliuca FW, Millman JR, Gurtler M, Segel M, Van Dervort A, Ryu JH, et al. Generation of functional human pancreatic beta cells in vitro. *Cell*. 2014;159(2):428–39.
5. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. *Nat Biotechnol*. 2014;32(11):1121–33.
6. Russ HA, Parent AV, Ringler JJ, Hennings TG, Nair GG, Shveygert M, et al. Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro. *EMBO J*. 2015;34(13):1759–72.
7. Farah S, Doloff JC, Muller P, Sadraei A, Han HJ, Olafson K, et al. Long-term implant fibrosis prevention in rodents and non-human primates using crystallized drug formulations. *Nat Mater*. 2019;18(8):892–904.
8. Vegas AJ, Veisheh O, Doloff JC, Ma M, Tam HH, Bratlie K, et al. Combinatorial hydrogel library enables identification of materials that mitigate the foreign body response in primates. *Nat Biotechnol*. 2016;34(3):345–52.
9. Vegas AJ, Veisheh O, Gurtler M, Millman JR, Pagliuca FW, Bader AR, et al. Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. *Nat Med*. 2016;22(3):306–11.
10. Sharivkin R, Walker MD, Soen Y. Proteomics-based dissection of human endoderm progenitors by differential cell capture on antibody array. *Mol Cell Proteomics*. 2012;11(9):586–95.
11. Sharivkin R, Walker MD, Soen Y. Functional proteomics screen enables enrichment of distinct cell types from human pancreatic islets. *PLoS One*. 2015;10(2):e0115100.
12. Tannenbaum SE, Turetsky TT, Singer O, Aizenman E, Kirshberg S, Ilouz N, et al. Derivation of xeno-free and GMP-grade human embryonic stem cells—platforms for future clinical applications. *PLoS*

One. 2012;7(6):e35325.

13. D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol.* 2005;23(12):1534–41.
14. Agulnick AD, Ambruzs DM, Moorman MA, Bhoumik A, Cesario RM, Payne JK, et al. Insulin-Producing Endocrine Cells Differentiated In Vitro From Human Embryonic Stem Cells Function in Macroencapsulation Devices In Vivo. *Stem Cells Transl Med.* 2015;4(10):1214–22.
15. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol.* 2006;24(11):1392–401.
16. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol.* 2008;26(4):443–52.
17. Schulz TC, Young HY, Agulnick AD, Babin MJ, Baetge EE, Bang AG, et al. A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. *PLoS One.* 2012;7(5):e37004.
18. Hrvatin S, Deng F, O'Donnell CW, Gifford DK, Melton DA. MARIS: method for analyzing RNA following intracellular sorting. *PLoS One.* 2014;9(3):e89459.
19. Rezanian A, Bruin JE, Xu J, Narayan K, Fox JK, O'Neil JJ, et al. Enrichment of human embryonic stem cell-derived NKX6.1-expressing pancreatic progenitor cells accelerates the maturation of insulin-secreting cells in vivo. *Stem Cells.* 2013;31(11):2432–42.
20. Taylor BL, Liu FF, Sander M. Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells. *Cell Rep.* 2013;4(6):1262–75.
21. Nostro MC, Sarangi F, Ogawa S, Holtzinger A, Corneo B, Li X, et al. Stage-specific signaling through TGFbeta family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. *Development.* 2011;138(5):861–71.
22. Kelly OG, Chan MY, Martinson LA, Kadoya K, Ostertag TM, Ross KG, et al. Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells. *Nat Biotechnol.* 2011;29(8):750–6.
23. Katsuta H, Akashi T, Katsuta R, Nagaya M, Kim D, Arinobu Y, et al. Single pancreatic beta cells co-express multiple islet hormone genes in mice. *Diabetologia.* 2010;53(1):128–38.
24. Cataldo LR, Mizgier ML, Bravo Sagua R, Jana F, Cardenas C, Llanos P, et al. Prolonged Activation of the Htr2b Serotonin Receptor Impairs Glucose Stimulated Insulin Secretion and Mitochondrial Function in MIN6 Cells. *PLoS One.* 2017;12(1):e0170213.
25. Baron M, Veres A, Wolock SL, Faust AL, Gaujoux R, Vetere A, et al. A Single-Cell Transcriptomic Map of the Human and Mouse Pancreas Reveals Inter- and Intra-cell Population Structure. *Cell Syst.* 2016;3(4):346–60. e4.
26. Veres A, Faust AL, Bushnell HL, Engquist EN, Kenty JH, Harb G, et al. Charting cellular identity during human in vitro beta-cell differentiation. *Nature.* 2019;569(7756):368–73.

27. Kaido T, Yebra M, Cirulli V, Montgomery AM. Regulation of human beta-cell adhesion, motility, and insulin secretion by collagen IV and its receptor alpha1beta1. *J Biol Chem.* 2004;279(51):53762–9.

28. Song J, Millman JR. Economic 3D-printing approach for transplantation of human stem cell-derived beta-like cells. *Biofabrication.* 2016;9(1):015002.

29. Bruin JE, Erener S, Vela J, Hu X, Johnson JD, Kurata HT, et al. Characterization of polyhormonal insulin-producing cells derived in vitro from human embryonic stem cells. *Stem Cell Res.* 2014;12(1):194–208.

Additional File

Additional file 1: Table S1. Additives to basal medium in the course of the seven-stage differentiation (30 to 44 days). Additional file 2: Table S2. Basal media composition and indicated additions to one liter during differentiation Additional file 3: Table S3. List of the qPCR primers. Additional file 4: MARIS: Method for Analyzing RNA following Intracellular Sorting Additional file 5: Table S4. List of antibodies used. Additional file 6: Table S5. Gene expression in ILC cells selected for binding to CD49A antibodies. Additionall file 7: Table S6. CD26 and other markers identified by FCCS

Figures

Fig. 1

Figure 1

Cell populations in Islet-like clusters at the end of the differentiation process. A. Protein immunostaining of an ILC for human C-peptide (green), Glucagon (purple), PDX1 (red, nuclear stain). B. Percentage of ILC cells stained by antibodies to indicated proteins, calculated from flow cytometry (FACS) analysis of a representative ILC preparation (antibodies listed in Table 4s). C. Flow-cytometry (FACS) analysis of dissociated total ILC cells, fixed and stained for human C-peptide (vertical axis) and Nkx6.1 (horizontal axis). Q3 is the double negative fraction. For the percentage of cells in each fraction, see Tables 1,2. D. The method for analyzing RNA after intracellular staining (MARIS) was applied to ILC cells sorted by preparative FACS using antibodies to human c-peptide and NKx6.1 antibodies. From each of the subpopulations (as in C), RNA was extracted and expression of indicated genes was measured by qPCR. Results calculated relative to the non-sorted total cells taken as 1.

Fig. 2

Figure 2

Functional Cell-Capture Screening on antibody array Identifies CD49A as beta cell marker. A. ILC cells attaching to CD49A and CD99 antibodies show a high proportion of Insulin-positive cells, while CD66c and CD73 antibody attached cells do not express insulin. Dissociated ILC cells were spread on arrays of 233 antibodies against cell-surface proteins and after incubation were reacted with anti-insulin antibodies and Cy-5 labelled secondary antibodies. The proportion of stained cells in the total cell captured was evaluated B. FACS plot of cells selected for binding to the CD49A antibody, and further stained for human C-peptide, Nkx6.1 and CD49A, shows that CD49A positive cells (blue) are mainly in the C-peptide/Nkx6.1 double positive fraction. C. qPCR analysis of RNA extracted from ILC cells fractionated by MACS shows that the cells binding to CD49A antibodies (CD49A enriched) have higher Insulin, UCN3 and mainly MAFA expression. Results calculated in comparison to cells prior to MACS taken as 100%.

Fig. 3

Figure 3

Normalization of glycemia in diabetic C57BL/6 mice and correlation with human c-peptide secretion. A. The blood glucose levels of diabetic mice are normalized for more than 100 days by implantation of hESC-derived ILC cells. The graph shows the average of blood glucose levels + SEM in STZ-treated mice implanted IP. with TMTD-MVG alginate encapsulated hES-derived ILC cells (solid line: 5 mice implanted with 2.2 million per mouse of cells at day 30 of differentiation; dotted line: non-implanted mice). Diabetes was induced by 4 daily injections of STZ at dose of 50 mg/kg and implantation done 15 days after (day 0). B. Higher C-peptide correlates with lower glycemia. In the same experiment, the blood glucose levels of two individual mice (solid lines) are compared to the levels of human C-peptide (broken lines) in the blood of the same animal (blue or red line respectively).

Fig. 4

Figure 4

Glycemia of diabetic mice is reduced by CD49A positive ILC cells but not by CD49A negative cells. A. C-peptide concentration levels in blood of mice implanted with CD49A enriched ILC cells is much higher than that in blood of mice implanted with CD49A depleted ILC cells, and also higher than that in mice implanted with intact ILC (Unsorted). The ILC cells at day 35 of differentiation were purified by two rounds of binding to a MACS column with CD49A antibodies, then reaggregated for 2 days, encapsulated in TMTD-MVG alginate (1.9 million per mouse), and implanted IP. to C57BL/6 mice two days after a single injection of STZ (150 mg/kg). Total unsorted ILC were similarly implanted. Blood C-peptide was measured 7 days post-implantation in the three groups of mice after overnight food deprivation (t=0, blue) and 30 minutes after IP injection of 2g/kg glucose (t=30, red). Average blood C-peptide level in pmol/L + SEM are shown for each group (n=8). The glucose-dependent C-peptide levels differ significantly, with two-tailed Student's t-test $p=0.039$ between Total ILC and CD49A enriched fraction and $p=0.008$ between the CD49A enriched and CD49A depleted fractions. B. Flow cytometry of ILC cells immunostained for C-peptide and Nkx6.1 showing the disappearance of the double positive population in the CD49A depleted MACS fraction (right panel) relatively to the CD49A-enriched fraction (left panel). C. ILC cells selected for the CD49A marker function in vivo to reduce glucose concentration in blood of diabetic mice. Average blood glucose + SEM of C57BL/6 mice treated with 150mg/kg STZ and implanted two days later with cells fractionated by MACS as described in A. Implantation was with the CD49A enriched MACS fraction (green squares), with the CD49A depleted fraction (dark blue triangles), or intact non-sorted cells (red dots). Controls non-implanted are shown (dotted blue line).

Fig. 5

Figure 5

CD26 depletion followed by CD49A selection eliminates the C-peptide+ fraction lacking Nkx6.1 and increases the C-peptide/NKX6.1 double positive fraction. Flow cytometry of ILC cells stained with C-peptide (vertical axis) and Nkx6.1 (horizontal axis) showing the four subpopulations (Fig. 1C). In the upper panel, non-sorted ILC cells were also stained for CD26 (blue). After selection by MACS with CD26 antibodies (middle two panels), the CD26 depleted cells have lost the C-peptide only fraction (right panel). After subsequent selection of the CD26 depleted fraction by MACS with CD49A antibodies (lower two panels), the C-peptide/Nkx6.1 DP fraction is increased (lower left panel with CD49A stain in blue). The percentage of cells in the four FACS fractions at each step of the double selection is given in Table 2. The total number of cells at each step is shown next to the panels.

Fig. 6

Figure 6

Improved activity of CD26 depleted/CD49A enriched ILC to normalize glycemia in diabetic mice. A. CD26 depleted/CD49A enriched ILC cells (green squares) implanted in diabetic C57BL/6 mice, reduce blood glucose better than non-sorted cells (red circles) or than CD26 depleted/CD49A depleted cells (black triangles). After a first MACS with CD26 antibodies, the CD26- flow-through cells were separated by MACS with CD49A antibodies. The non-sorted and the sorted cell fractions were reaggregated into ILC prior to micro-encapsulation in TMTD-MVG alginate and, from each, a dose of 1 million cells was implanted IP. in mice which were diabetic for 45 days after an injection of STZ (150 mg/kg). Average blood glucose + SEM is shown. B. In the same mice, blood C-peptide was measured at indicated days, after overnight food deprivation (t=0) and 30 minutes after IP injection of 2 g/kg glucose (t=30). Average blood levels of human C-peptide in pmol/L + SEM are shown for mice with implants of ILC either non-sorted (red), CD26 depleted/CD49A enriched (green) or CD26 depleted/CD49A depleted (blue). C. Intraperitoneal Glucose Tolerance Test (IPGTT) shows that CD26 depleted/CD49A enriched ILC cells (green squares) reduce more the diabetic state than non-sorted ILC cells (red circles). In this experiment, C57BL/6 mice injected for 4 days with 50 mg/kg STZ were implanted after 18 days with the sorted and non-sorted ILC (as in A). On day 46 after implantation the mice were injected IP. with 2g/kg glucose and blood glucose was measured at different times for a 2 hours period. Average blood glucose (+ SEM) is shown. The AUC with the CD26 depleted/CD49A enriched cells was 40 % lower than that with the non-sorted re-aggregated cells (p=0.046).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [MolakandovK.Additionalfiles.docx](#)