Supporting Information

Pennarossa et al. 10.1073/pnas.1220637110

SI Experimental Procedures

Quantitative PCR. RNA was extracted with the TaqManGene Expression Cells to Ct kit (Applied Biosystems), and DNase I (Invitrogen) was added in lysis solution at 1:100 concentration, as indicated by the manufacturer's instructions. The effective removal of genomic DNA from each RNA batch was then confirmed by performing a standard PCR amplification for β-actin, using genomic DNA as a positive control. Only negative samples were then reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen). Predesigned gene-specific primer and probe sets from TaqManGene Expression Assays (Applied Biosystems) were used for gene study (Table S1). PCR runs and fluorescence detection were carried out in a 7500 Real-Time PCR System (Applied Biosystems). β-actin was used as internal standard.

For each individual gene, the number of amplification cycles for the fluorescent reporter signal to reach a common threshold value (Ct) were estimated and then normalized against the Ct value obtained for β-actin of the same sample to give the ΔCt value. Gene expression levels are reported with the highest expression set to 1 and all other times relative to this.

Immunocytochemistry. Cells were fixed, permeabilized, and treated with blocking solution [PBS containing 5% (vol/vol) not immune serum]. Primary antibodies and their working dilutions are listed in Table S2. Cells were incubated with suitable secondary antibodies (Alexa Fluor, Invitrogen) for 45 min. Nuclei were stained with DAPI (Sigma). Samples were observed under a Nikon Eclipse TE200 microscope. When cells formed spherical structures, these were dissociated and attached to slides, using a cytocentrifuge (Cytospin 4, Thermo Shandon).

Cell Counting. The number of cells immunopositive for SRY-related HMG-box transcription factor SOX17 (Sox17) hepatocyte nuclear factor 3-beta (Foxa2), paired box protein Pax-6 (Pax6), insulin gene enhancer protein ISL-1 (Isl1), pancreatic and duodenal homeobox protein 1 (Pdx1), and homeobox protein Nkx-6.1 (NKX6.1) was counted in 15 randomly selected fields at 200× total magnification. A minimum of 600 cells were counted in three independent replicates. The numbers of positively stained cells were expressed as a percentage of the total cell counted.

Western Blots. Cells were collected at time 0 (T_0), after 18 h exposure to the cytidine analog 5-azacytidine (5-aza-CR; post 5-aza-CR) and on days 10, 14, 20, 30, and 42 of pancreatic induction. They were lysed, and their constitutive proteins were extracted using a ReadyPrep Protein Extraction Kit (Bio-Rad). Protein concentration was assessed by Coomassie Blue-G Dye-binding methods (1). Aliquots of 100 μg were prepared and resuspended in sample buffer (1:1) consisting of 4% (wt/vol) SDS, 10% 2-mercaptoethanol, 20% (wt/vol) glycerol, 0.004% bromophenol blue, and 0.125 M Tris–HCl at pH 6.8. Equal samples were loaded and electrophoresed on a SDS-polyacrylamide gel (20% for low-molecular weight proteins and 10% for other proteins). Proteins were then transferred onto 0.2- and 0.45-μm pore size nitrocellulose filters (Hybond-C Extra, Amersham), respectively, for low-molecular weight proteins and other proteins. The membrane was probed with primary antibodies listed in Table S2. Protein bands were visualized by the WesternBreeze chemiluminescent kit (Invitrogen). Densitometric analysis was performed with Quantity One 1-D Analysis Software (Bio-Rad).

Cell Growth Curve, Apoptotic and Proliferation Index. Growth curve assessment was carried out by plating 1.5×10^5 cells/well in 24-well multidishes (Nunc). Cell number was counted using Hycor KOVA Glasstic (Fisher). Cell viability was determined by trypan blue dye exclusion assay. Each time point was assessed in triplicate.

Apoptotic index was evaluated by staining treated cells with TUNEL, using a commercially available kit (Roche) and following the manufacturer's instructions.

The cell proliferation index was assessed by staining treated cells with a Ki67-specific antibody (Table S2), using the same immunocytochemistry protocol described earlier. A minimum of 200 cells for each point of each biological replicate were counted.

Karyotype. Thirty metaphases in each of three independent replicates were analyzed. After colcemid treatment and Giemsa staining (KarioMAX Giemsa), metaphases were examined under a Leica HC microscope equipped with a digital camera Leica DC250. Images were analyzed using Leica CW4000 Karyo software.

Assessment of C-Peptide Release. To avoid possible confounding effects by the insulin content of the culture medium, the functional activity of pancreatic converted cells (PCCs) was evaluated measuring C-peptide release in supernatants obtained from cell on days 42 and 102 of pancreatic induction. Culture medium was removed and cells were rinsed with PBS and then stimulated for 1 h and 24 h with 20 mM D-glucose or L-glucose (final concentration), respectively, in DMEM supplemented with 10% (vol/vol) FBS (Gibco) and 2 mM glutamine (Sigma) (2). Glucose-dependent C-peptide release was assessed with a Human C-peptide ELISA Kit (EIAab), following the manufacturer's instructions. Values were normalized against DNA content, measured using PicoGreen (Invitrogen).

Flow Cytometry. On day 42 of culture, PCCs were dissociated with 0.25% trypsin-EDTA (Invitrogen) and Accutase (Innovative Cell Technologies) at 37°C for 10–15 min. Cells were washed and fixed with 2% (wt/vol) paraformaldehyde in PBS at room temperature for 45 min and permeabilized with 0.2% TRITON X-100 in PBS for 15 min. Before incubation with primary antibodies, pellets were resuspended in blocking solution [5% (wt/vol) BSA and 3% (vol/vol) goat serum in PBS] and incubated for 20 min. Cells were incubated with an anti-C peptide antibody (Table S2) at room temperature for 30 min, washed with PBS, and stained with appropriate secondary antibody conjugated (1:500, Alexa Fluor 488, Invitrogen) at room temperature for 30 min. Cells were then washed and resuspended in PBS. Samples incubated with primary isotypic antibody were used as a control. Flow cytometry was carried out with a FACS Canto II (BD Bioscience) and analyzed with BD FACSDiva v6.1.3 software.

DNA Methylation Analysis. Global DNA methylation was assessed as previously described (3, 4). DNA was extracted using an automatic extraction system with the Maxwell 16 LEV DNA Purification Kit (Promega), following the manufacturer's instructions. DNA concentration was assessed with NanoDrop 8000 (Thermoscientific). Aliquots of 0.8 ng total DNA were prepared in a total volume of 2 μL per sample and spotted onto nylon membranes (Hybond-N+, Amersham). Membranes were allowed to dry, UV-crosslinked for 1 min, and probed with a primary antibody against 5-methylcytidine (Table S2). Dots were visualized with a WesternBreeze chemiluminescent kit (Invitrogen). Signal intensity was quantified by densitometric analysis, using the Image J analysis software (National Institutes of Health). Assays were performed on the adult cell lines and on PCS-201-010 cells, in triplicate for each sample.

1. Read SM, Northcote DH (1981) Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Anal Biochem* 116(1): 53–64.
2. Josefsen K, et al. (1998) Glucose stimulation of pancreatic beta-cell lines induces expression and secretion of dynorphin. *Endocrinology* 139(10):4329–4336.
3. Segura-Pacheco B, et al. (2006) Global DNA hypermethylation-associated cancer chemotherapy resistance and its reversion with the demethylating agent hydralazine. *J Transl Med* 4:32.
4. Meeran SM, Patel SN, Li Y, Shukla S, Tollefsbol TO (2012) Bioactive dietary supplements reactivate ER expression in ER-negative breast cancer cells by active chromatin modifications. *PLoS ONE* 7(5):e37748.

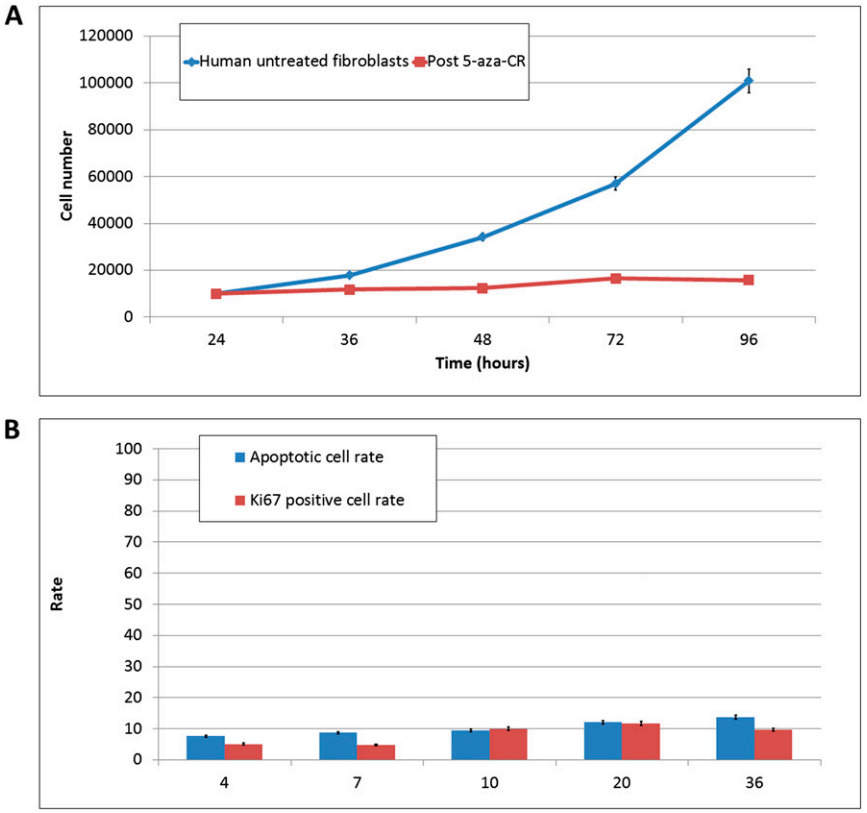

Fig. S1. Effect of 5-aza-CR exposure and pancreatic induction on human cell proliferation. (*A*) No difference in cell proliferation was observed between 5-aza-CR-exposed and untreated cells 24 and 36 h after the removal of 5-aza-CR. However, from the following 24 h, cells exposed to 5-aza-CR showed a sharply decreased proliferation rate. (*B*) During the differentiation process, the total cell number remained substantially stable as a result of similar proliferation and apoptotic indexes.

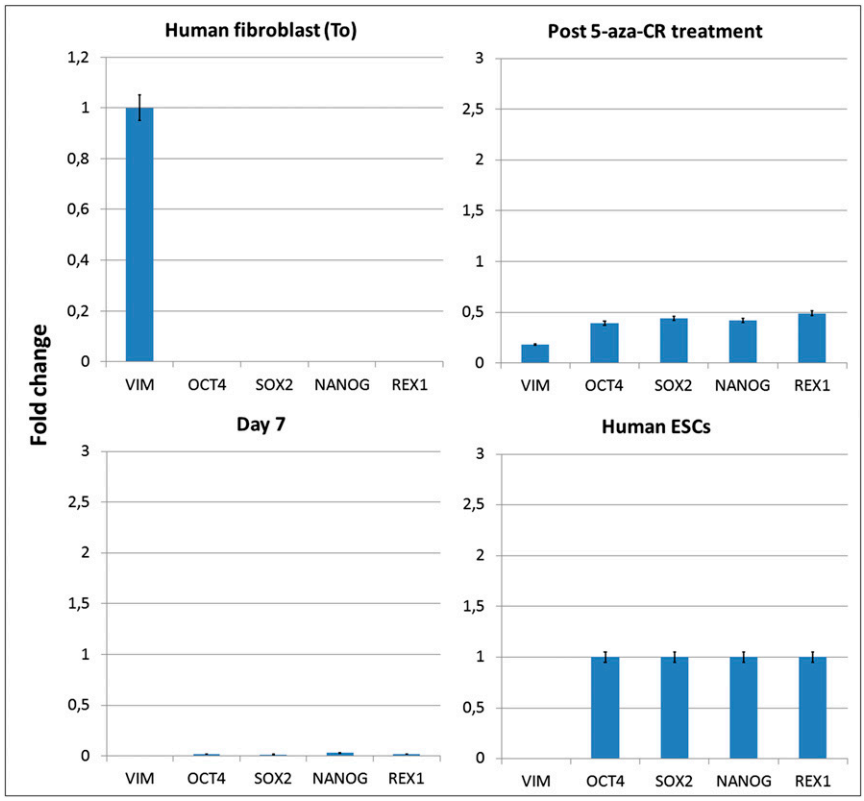

Fig. S2. Expression pattern of vimentin and pluripotency related genes in human fibroblasts after 5-aza-CR exposure. Untreated fibroblasts (T_0) expressed high levels of vimentin, a fibroblast-specific marker. Exposure to 5-aza-CR resulted in a sharp down-regulation of vimentin accompanied by the onset of pluripotency marker expression. After 7 d of culture in endocrine pancreatic induction medium, expression of vimentin and pluripotency genes was no longer detectable.

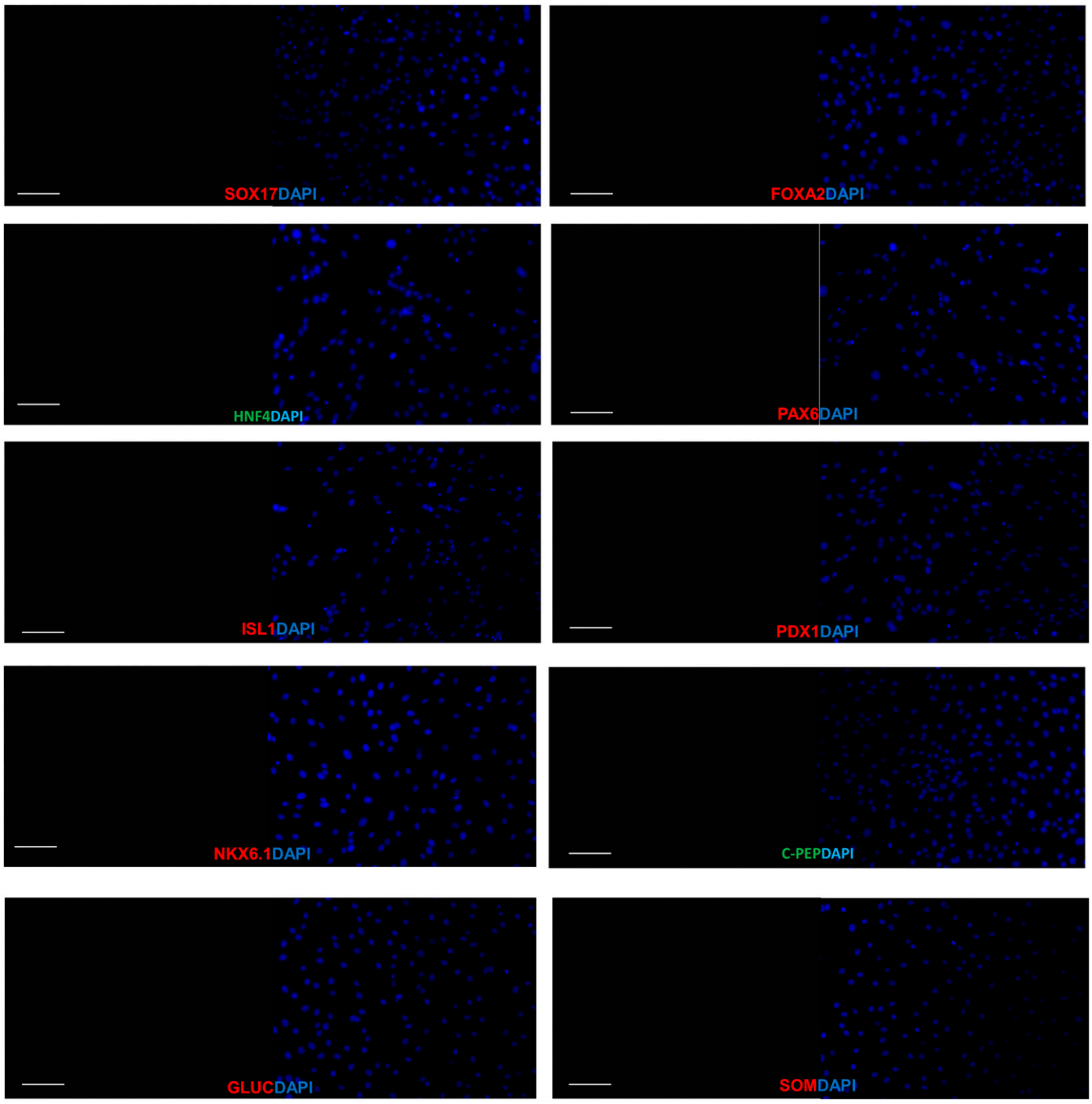

Fig. S3. Untreated fibroblasts do not express endoderm/pancreatic markers. Human fibroblasts examined before their exposure to 5-aza-CR (T_0) showed no signals of definitive endoderm (Sox17, Foxa2), primitive gut tube (Hnf4), endodermal precursors (Pax6, Isl1), posterior foregut (Pdx1), pancreatic endoderm (Nkx6.1), or pancreatic endocrine cell [C-peptide (C-PEP), glucagon (GLUC), somatostatin (SOM)] markers. (Scale bar, 100 μm.)

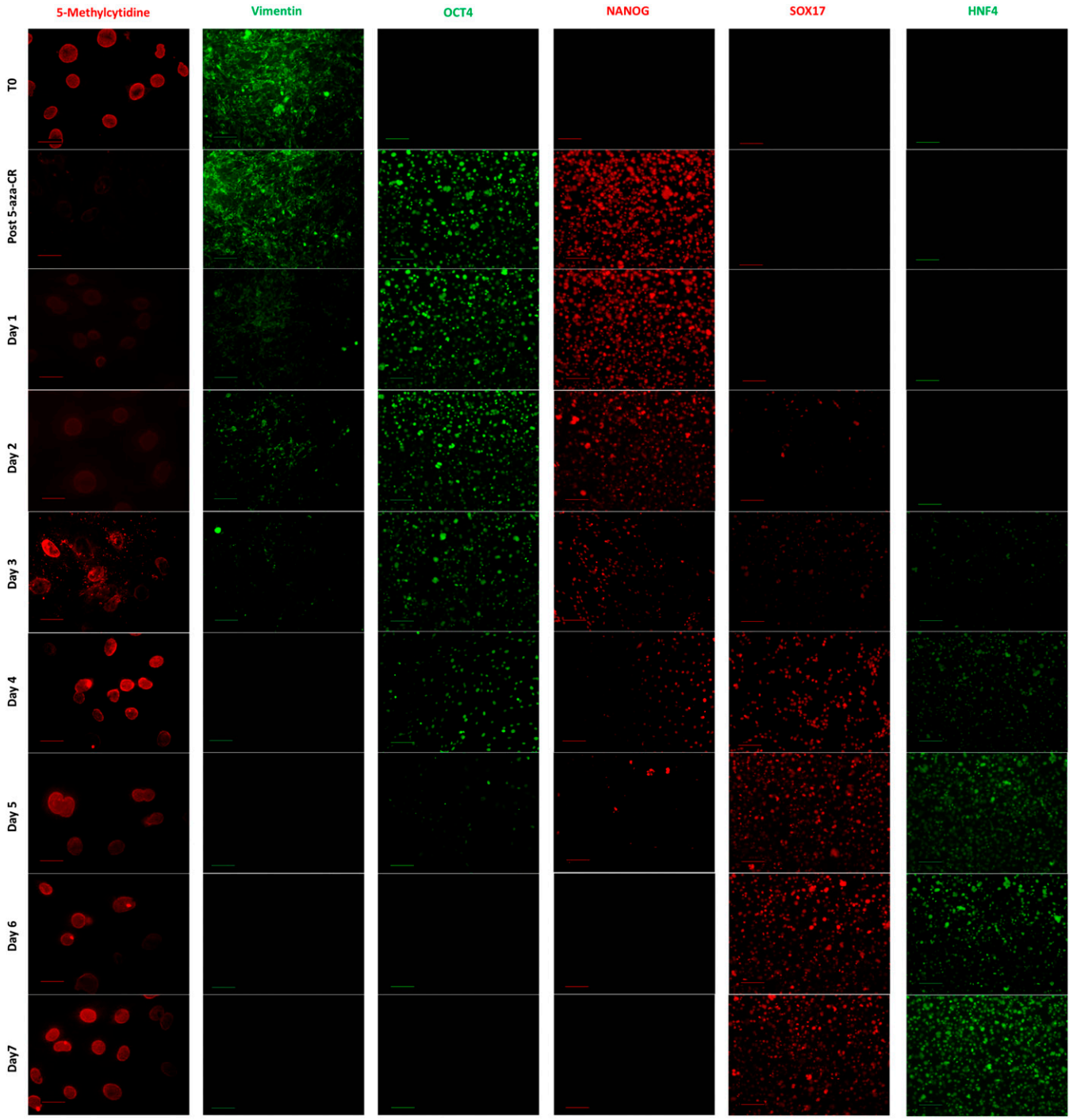

Fig. S4. Relationship between 5-methylcytidine immunostaining and expression of fibroblast (vimentin), pluripotency (Oct4, Nanog), and definitive endoderm/primitive gut tube (Sox17, hnf4) markers. Immunolabeling was performed on untreated fibroblasts (T_0) at the end of exposure to 5-aza-CR (post-5-aza-CR) and from day 1 to day 7 of pancreatic induction. (Scale bar, 20 μm for 5-methylcytidine and 100 μm for the rest.)

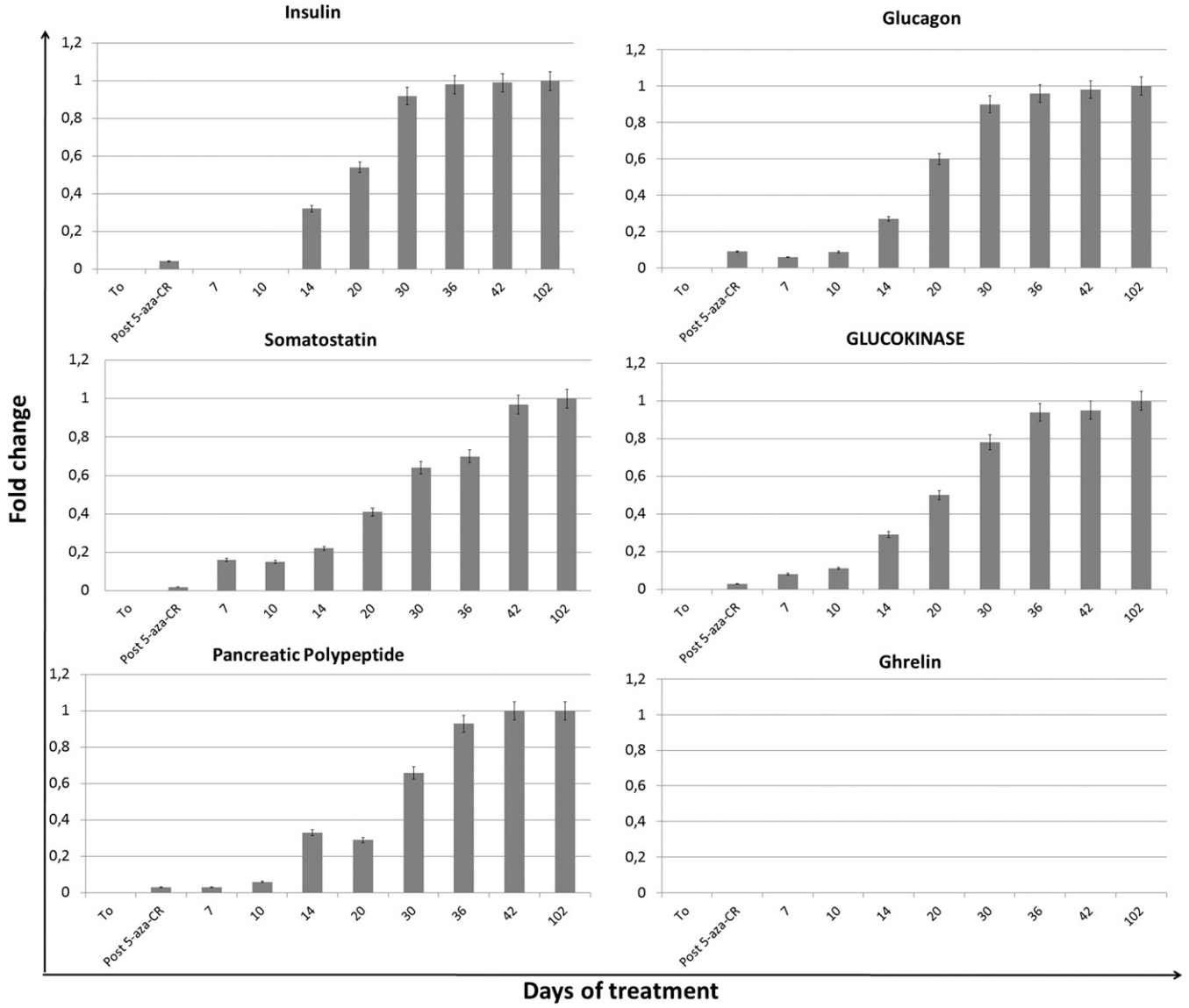

Fig. S5. Expression patterns of hormone and glucose sensor genes characteristic of mature endocrine pancreatic cells in human PCCs. All genes showed an expression level of physiological relevance. *Ghrelin* mRNA was the only one that could not be detected.

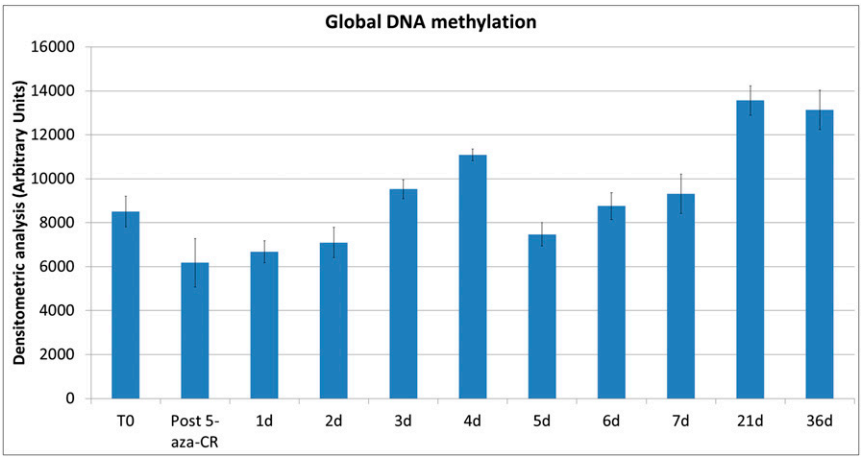

Fig. S6. Global methylation pattern of fibroblasts exposed to 5-aza-CR during their pancreatic differentiations. Histogram represents dot-blot signal intensity quantified by densitometric analysis using Image J analysis software (National Institutes of Health). Bars represents the mean ± SD of three independent replicates.

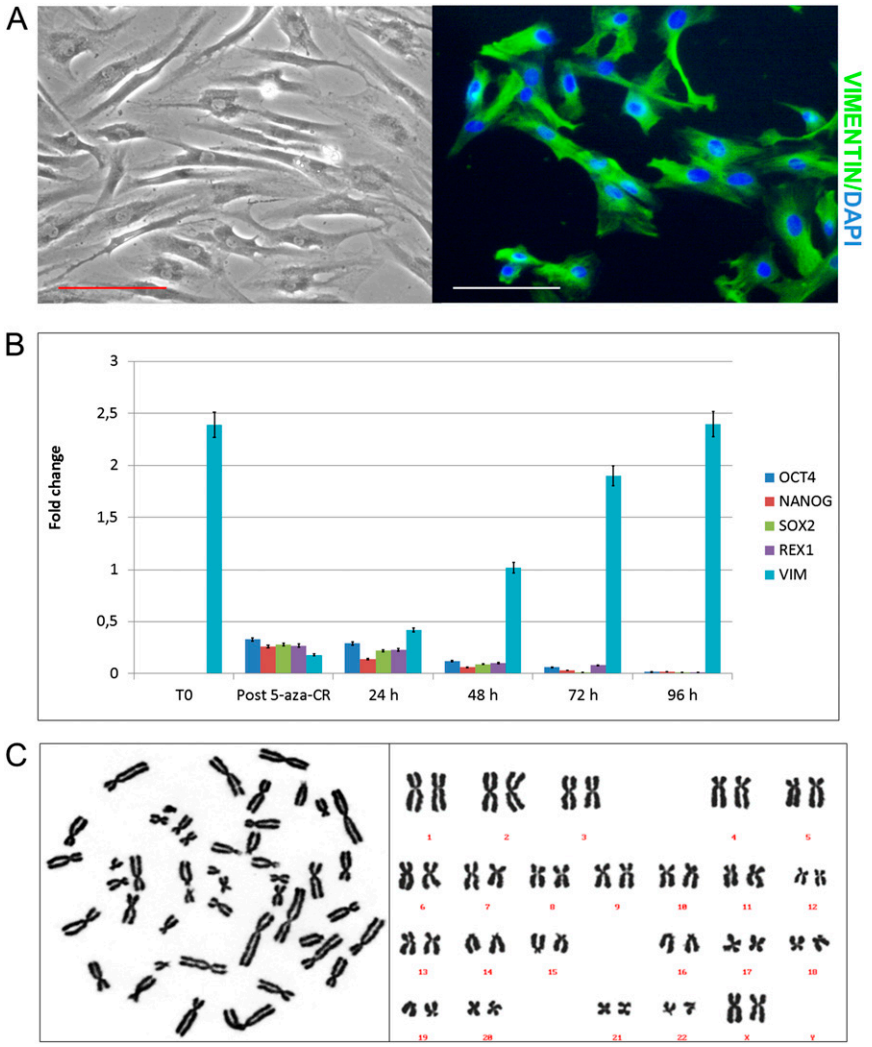

Fig. S7. The effect of 5-aza-CR is reversible, and cells maintain a normal karyotype. When post-5-aza-CR human fibroblasts were returned to standard culture medium they reverted to the initial morphology and resumed vimentin expression (*A*). (Scale bar, 200 μm.) Within 4 d, fibroblasts completely down-regulated pluripotency-related genes and expressed vimentin at the same level measured before exposure to 5-aza-CR (*B*). Cells maintained a normal karyotype for 102 d, the entire length of the experiment (*C*).

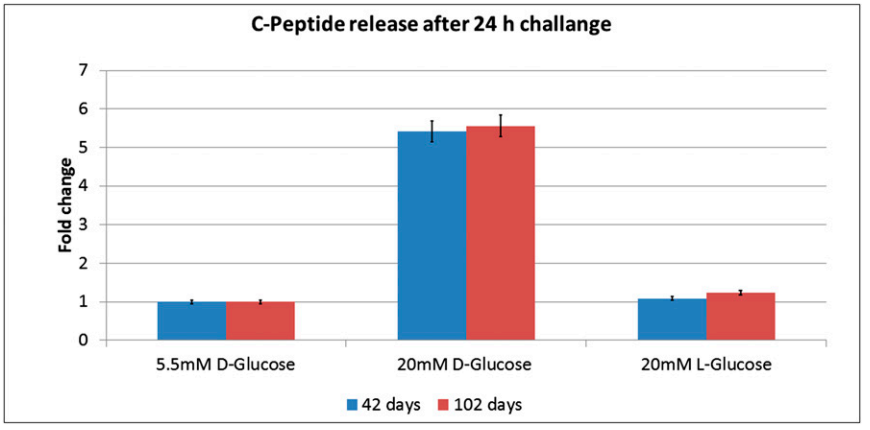

Fig. S8. In vitro functional response of human PCCs to a prolonged glucose stimulation. Quantification of C-peptide release in the culture medium in response to 20 mM D-glucose for 24 h at different time of culture. Glucose was able to stimulate secretion of C-peptide in the culture medium even after 102 d of culture. C-peptide release in the absence of glucose challenge was low and used as baseline reference value. Only baseline levels of C-peptide release were elicited by 20 mM L-glucose. Bars, mean ± SD of three independent replicates.

Table S1. List of primers used for quantitative PCR analysis of human cells

Gene	Description	Catalog no.
ACTB	*Actin,beta*	*Hs01060665_g1*
FOXA2	*forkhead box A2*	*Hs00232764_m1*
GHRL	*ghrelin/obestatin prepropeptide*	*Hs01074053_m1*
GCG	*glucagon*	*Hs01031536_m1*
GCK	*glucokinase (hexokinase 4)*	*Hs01564555_m1*
HNF1B	*HNF1 homeobox B*	*Hs01001602_m1*
HNF4A	*hepatocyte nuclear factor 4, alpha*	*Hs00230853_m1*
INS	*insulin*	*Hs02741908_m1*
ISL1	*ISL LIM homeobox 1*	*Hs00158126_m1*
MAFA	*v-maf musculoaponeurotic fibrosarcoma ncogene homolog A*	*Hs01651425_s1*
MAFB	*v-maf musculoaponeurotic fibrosarcoma oncogene homolog B*	*Hs00534343_s1*
NANOG	*Nanog homeobox*	*Hs02387400_g1*
NES	*nestin*	*Hs04187831_g1*
NEUROD	*neuronal differentiation 1*	*Hs00159598_m1*
NKX6.1	*NK6 homeobox 1*	*Hs00232355_m1*
OCT4	*POU class 5 homeobox 1*	*Hs00999632_g1*
ONECUT1	*one cut homeobox 1*	*Hs00413554_m1*
PAX6	*paired box 6*	*Hs00240871_m1*
PCSK1	*proprotein convertase subtilisin/kexin type 1*	*Hs01026107_m1*
PCSK2	*proprotein convertase subtilisin/kexin type 2*	*Hs01037347_m1*
PDX1	*pancreatic and duodenal omeobox 1*	*Hs00236830_m1*
PPY	*pancreatic polypeptide*	*Hs00358111_g1*
REX1	*ZFP42 zinc finger protein*	*Hs00399279_m1*
SOX2	*sex determining region Y-box 2*	*Hs01053049_s1*
SOX17	*sex determining region Y-box 17*	*Hs00751752_s1*
SST	*somatostatin*	*Hs00356144_m1*
VIM	*vimentin*	*Hs00185584_m1*

Table S2. List of antibodies and working dilutions used for immunocytochemical and western blot analysis

Antibody	Host species	Company	Cat. no.	ICC working dilution	WB working dilution
5-Methylcytidine	Mouse	Eurogentec	MMS-900P-B	1:500	1:500 (dot blot)
Ki67	Rabbit	Abcam	ab833	1:50	-
Vimentin	Mouse	Chemicon	MAB1687	1:200	-
Oct4	Rabbit	Chemicon	AB3209	1:50	-
Nanog	Goat	R&D System	AF1997	1:20	-
Sox17	Mouse	Abcam	ab84990	1:100	1:5000
Islet1	Rabbit	Abcam	ab20670	1:500	-
Islet1	Mouse	Abcam	ab86472	-	1:500
Foxa2	Mouse	Abcam	ab60721	1:100	1:500
Pax6	Rabbit	Abcam	ab5790	1:500	1:500
Pdx1	Rabbit	Abcam	ab47267	1:500	1:1000
Hnf4	Mouse	Abcam	ab41898	1:100	1:1000
Nkx6.1	Goat	Santa Cruz Biotechnology	sc-15030	1:100	1:500
Insulin	Mouse	Abcam	ab9569	-	1:500
C-peptide	Rabbit	Abcam	ab14181	1:100	1:100
Somatostatin	Rabbit	Abcam	ab103790	1:100	1:500
Glucagon	Mouse	Abcam	ab10988	1:100	-
Glucagon	Rabbit	Abcam	ab108426	-	1:2000
Gherelin	Rabbit	Santa Cruz Biotechnology	sc-50297	-	1:500
B-Actin	Mouse	Sigma	a5441	-	1:1000

ICC , immunocytochemical; WB, Western blot.