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Synthetic mRNA is a more reliable tool for the delivery of DNA-targeting proteins into the cell nucleus than fusion with a protein transduction domain

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Abstract

Cell reprogramming requires efficient delivery of reprogramming transcription factors into the cell nucleus. Here, we compared the robustness and workload of two protein delivery methods that avoid the risk of genomic integration. The first method is based on fusion of the protein of interest to a protein transduction domain (PTD) for delivery across the membranes of target cells. The second method relies on de novo synthesis of the protein of interest inside the target cells utilizing synthetic mRNA (syn-mRNA) as a template. We established a Cre/lox reporter system in three different cell types derived from human (PANC-1, HEK293) and rat (BRIN-BD11) tissues and used Cre recombinase to model a protein of interest. The system allowed constitutive expression of red fluorescence protein (RFP), while green fluorescence protein (GFP) was expressed only after the genomic action of Cre recombinase. The efficiency of protein delivery into cell nuclei was quantified as the frequency of GFP^+ cells in the total cell number. The PTD method showed good efficiency only in BRIN-BD11 cells (68%), whereas it failed in PANC-1 and HEK293 cells. By contrast, the syn-mRNA method was highly effective in all three cell types (29–71%). We conclude that using synthetic mRNA is a more robust and less labor-intensive approach than using the PTD-fusion alternative.

Introduction

Cell reprogramming is an emerging approach for treating an increasing number of human diseases [1]. Reprogramming factors, such as transcription factors, need to be delivered effectively into target cell nuclei. Delivery methods based on viral vectors or transposon systems are highly effective [2,3]. However, they carry inherent risks of unpredictable modifications of the

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target cell genome by random and irreversible integrations of exogenous DNA, which can cause insertional mutagenesis and carcinogenesis. Therefore, such approaches are not suitable for eventual clinical applications [4,5].

To avoid this limitation, alternative integration-free strategies have been developed. Direct application of recombinant proteins to cells is generally not feasible because most proteins do not cross cellular membranes. However, specialized protein domains that naturally facilitate transmembrane transport of polypeptides have been discovered [6] and harnessed as a novel protein delivery tool [7]. Dohoon et al. [8] successfully used a protein-based protocol to generate induced pluripotent stem cells (iPS), albeit with a lower efficiency in comparison to virus-based protocols [9]. Another promising strategy relies on the *de novo* synthesis of cargo proteins inside the target cell, where the structural information is provided by synthetic mRNA [10,11]. Warren et al. used this approach to successfully reprogram somatic cells into iPS, and subsequently to terminally differentiated myogenic cells [12]. To the best of our knowledge, although a number of delivery methods have been compared [13], a direct comparison between the two integration-free methods utilizing either the protein transduction domain (PTD) or synthetic mRNA has not been performed.

The aim of the present study was to provide such a comparison using diverse cell lines. The focus of our laboratory is the reprogramming of cells of pancreatic origin [14]. We selected the human pancreatic cancer cell line PANC-1 [15], which was previously used for cell fate manipulation and reprogramming using other methods [16], and the rat insulinoma cell line BRIN-BD11 [17], which represents terminally differentiated cells with regulated secretory pathways. Additionally, we chose the human embryonic kidney cell line HEK293 [18], which is of neuronal origin [19] and has been used extensively for producing exogenous proteins in research and industry [20]. Cre recombinase is an enzyme not normally present in mammalian cells. It has the capacity to specifically rearrange nuclear DNA in conjunction with the targeting sequence loxP [21]. The delivery of Cre recombinase to cell nuclei can be unequivocally detected by monitoring phenotypic effects of the irreversible, site-specific recombination of genomic DNA, such as small deletions [22, 23]. Using Cre recombinase as a model of the cargo protein, we designed and prepared PTD- and mRNA-based Cre recombinase constructs. We engineered three Cre-sensitive cell lines utilizing green fluorescent (GFP) and red fluorescent proteins (RFP) as the reporter system. Using this model, we compared the efficiency, reliability, and the workload of the two respective methods.

Materials and methods

Experimental design

Three cell lines were genetically modified using a DNA expression cassette that encoded red and green fluorescent proteins placed downstream of a strong constitutive promoter (Fig 1A). Coding sequences of RFP and GFP were separated by two stop codons flanked by two parallel recognition sites for Cre recombinase (Fig 1A, S1 Fig). Constitutively expressed RFP was used to prepare Cre-responsive cell clones. Cre recombinase-sensitive expression of GFP was used to detect the activity of the recombinase delivered into the cell nuclei. 2A self-cleaving peptide was employed to separate RFP and GFP from a bicistronic product (Supplementary information 1) [24]. Two delivery methods were tested: the purified recombinant fusion protein (PTD-Cre, Fig 1B) and the synthetic mRNA construct (syn-mRNA-Cre, Fig 1C). The efficiency of the Cre protein delivery was quantified using flow cytometry (Fig 1D). The amount of intracellular Cre protein was compared by western blot (Fig 1E).

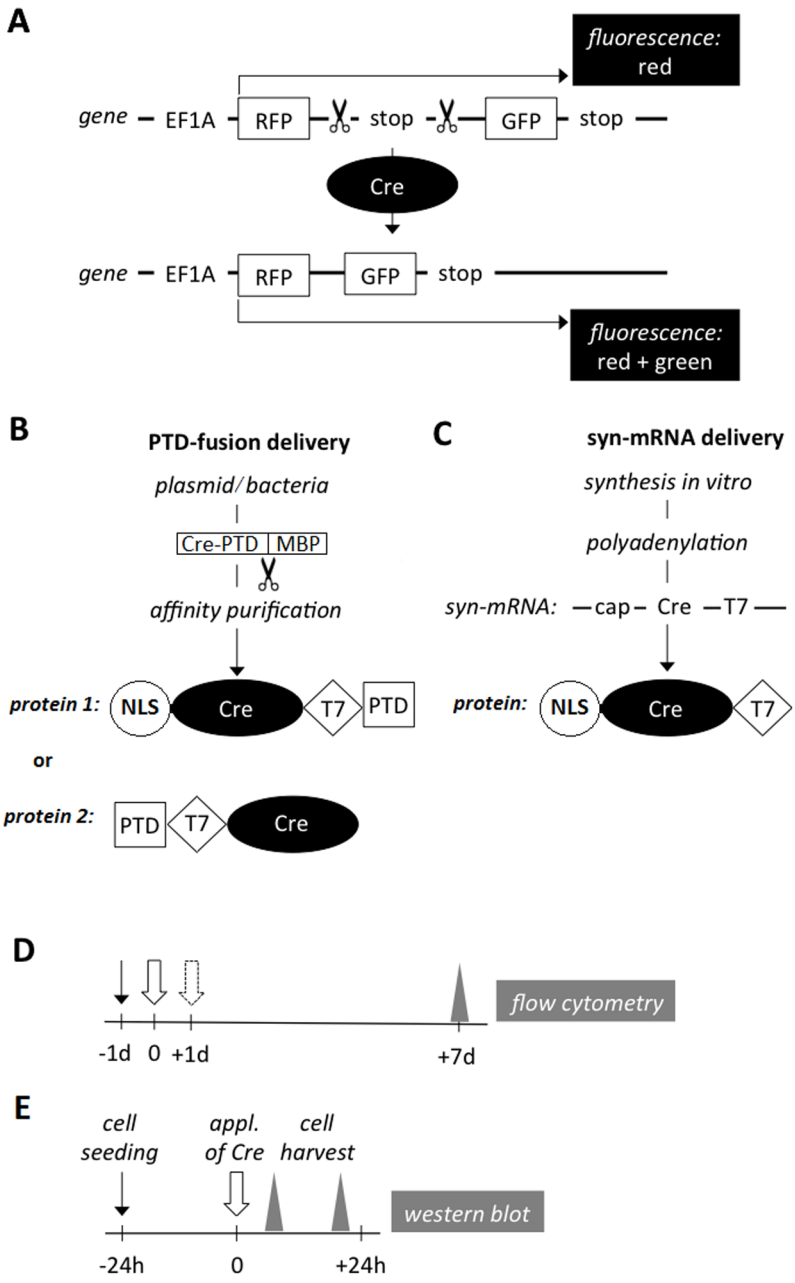

Fig 1. Experimental design. (A) RFP/GFP expression cassette inserted randomly into the genomes of the target cells. In these Cre-sensitive cells, the GFP expression was dependent on the delivery of functional Cre protein into cell nuclei. Scissors, parallel loxP sequences; Cre, Cre recombinase; stop, two stop codons. (B) The PTD-fusion proteins were produced in bacteria and purified in three steps. (C) The syn-mRNA (cap-NLS-T7-Cre) was synthesized *in vitro*. aa, number of amino acids; NLS, Nuclear localization signal (9 aa); Cre, Cre recombinase (343 aa); PTD, Protein transduction domain of HIV TAT (11 aa); T7, T7-tag (11 aa). Time frames of the flow cytometry (D) and western blot (E) analyses. Black arrows, cell seeding; white arrows, administration of Cre recombinase; dashed white arrow, second administration of Cre recombinase; grey triangles, harvesting of cells for analysis.

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Generation and culture of Cre-responsive cell lines

An expression cassette was designed (Fig 1A, S1 Fig) consisting of an RFP-loxP-stop-loxP-GFP sequence under the EF1a promoter and the hygromycin resistance selection marker. This was cloned into the piggyBac vector pD557-RA (DNA2.0, Menlo Park, CA) between the restriction sites *Bmt*I *and Bam*HI. A total of 3 μg of the piggyBac construct was combined with TransIT-X2 (Mirus, Madison, WI) at a 1:2 ratio and added to the respective cell lines PANC-1, BRIN-BD11 and HEK293 (all from Sigma-Aldrich, St. Louis, MO). After 14 days, cells were detached with 0.63% trypsin (Sigma-Aldrich). Clonal populations of the genetically modified cells were obtained by the sorting of single-cell suspensions to one cell per well, based on the RFP fluorescence signal using a BD Influx Cell sorter (Becton Dickinson, Franklin Lakes, NJ). The Cre-responsive cells were given the names fl-PANC, fl-BRIN and fl-HEK.

Cells were cultured in ventilated flasks (Corning, Corning, NY) at 37˚C, atmospheric O_2 and 5% CO_2. The PANC-1 cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 25 U/ml penicillin, 25 μg/ml streptomycin, 1 mM L-glutamine, and 1% Glutamax. The BRIN-BD11 cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 25 U/ml penicillin, 25 μg/ml streptomycin, 1 mM L-glutamine, and 1% Glutamax. HEK293 cells were cultured in MEM medium supplemented with 10% FBS (both from Sigma-Aldrich), 25 U/ml penicillin, 25 μg/ml streptomycin, 1 mM L-glutamine, 1% Glutamax, and 1% non-essential amino acids solution (all from Thermo Fisher Scientific, Waltham, MA).

Production of PTD-Cre recombinase protein

Two variants of PTD-Cre protein were prepared in a native state (Fig 1B). Correct folding of Cre recombinase was ensured by its fusion to the maltose binding protein (MBP), which functioned as a molecular chaperone [25]. MBP was subsequently cleaved off by TEV protease and removed by purification. The design of PTD-Cre1 (S2 Fig) was based on AAV-pgk-Cre, a kind gift from Patrick Aebischer (Addgene plasmid # 24593). The design of PTD-Cre2 (S3 Fig) was based on pTAT-Cre [26] (Addgene plasmid # 35619). The respective DNA constructs were cloned into the pMALc5x plasmid (New England Biolabs, Ipswitch, MA) between the restriction sites *Sac*I and *Bam*HI using the In-fusion HD Cloning Kit (Clontech, Mountain View, CA). NEB express competent *E. coli* (New England Biolabs) were transformed with the plasmids and cultured in LB medium (Carl Roth, Karlsruhe, DE) with 2% glucose (Sigma-Aldrich) and 50 μg/ml ampicillin (Serva, Heidelberg, DE) at 37˚C, and agitated at 260 rpm. Protein expression was induced by 0.17 mM IPTG (Sigma-Aldrich) for 4 hours at 30˚C, after which the bacterial cells were sonicated in a MBP-binding buffer containing 50 μg/ml DNase (Roche, Rotkreuz, CH) and 1 mg/ml lysozyme (Serva). The supernatant was placed on a MBPTrapHP column (GE Healthcare Life Science, Little Chalfont, UK) with MBP-specific affinity, and the purified MBP-PTD-Cre-T7 protein was eluted. Next, the MBP was cleaved off by the addition of TEV protease and separated from PTD-Cre-T7 by a second round of the MBPTrapHP column purification. Finally, PTD-Cre-T7 protein was quantified using the BCA protein assay (Thermo Fisher Scientific) and transferred to the fresh culture media using a 10 kDa Amicon filter (Merck Millipore, Darmstadt, DE). The PTD-Cre protein aliquots (53 μM) were stored at -20˚C for one month.

Production of syn-mRNA-Cre

The syn-mRNA-Cre construct (Fig 1C) was synthesized *in vitro* using the T7 mScript Standard mRNA Production System (CELLSCRIPT, Madison, WI) and 2 μg of purified DNA template. The template DNA was designed (S4 Fig) and synthesized using AAV-pgk-Cre, a kind gift

from Patrick Aebischer (Addgene plasmid # 24593). A custom ribonucleotide blend comprised of 3′-0-Me-m7G(5′)ppp(5′)G ARCA cap analog, pseudouridine triphosphate, 5-methylcytidine triphosphate (TriLink Biotechnologies, San Diego, CA), ATP, and GTP (New England Biolabs) was prepared. The final reaction mixture (20 μL), containing 6 mM ARCA cap analog, 3.0 mM ATP, and 1.5 mM of each of the other nucleotides, was incubated for 1 hour at 37˚C. The DNA template was then degraded by Turbo DNase (Life Technologies, Grand Island, NY), which was removed by ammonium acetate precipitation. The residual 5′-triphosphates were degraded by 2 hour incubation at 37˚C with Antarctic phosphatase (New England Biolabs), which was removed by ammonium acetate precipitation. After a 2 hour treatment at 37˚C with yeast Poly(A) Polymerase (Affymetrix, Santa Clara, CA), the polyadenylated synthetic mRNA was finally repurified with a MEGAclear Transcription Clean-Up Kit, diluted with RNAsecure Resuspension Solution and quantified with a Qubit fluorometer (all from Thermo Fisher Scientific).

Administration of PTD-Cre and syn-mRNA-Cre

The Cre-responsive cell lines were grown for several days in their respective culture media, which were changed at various degrees of confluence (to account for subsequent growth) prior to the addition of PTD-Cre or syn-mRNA-Cre. The purified PTD-Cre protein, originally dissolved in the respective culture media, was added directly to the cells at three final serial dilutions (15, 7.5 and 3.75 μM). The syn-mRNA-Cre was added at three final decimal dilutions (2.1, 0.21, and 0.021 nM) in Lipofectamine/Opti-MEM transfection reagent. Lipofectamine messenger MAX transfection reagent was first diluted with Opti-MEM medium at a 1:33 volume ratio. Then, syn-mRNA-Cre diluted in Opti-MEM (all from Thermo Fisher Scientific) was added at a 1:1 volume ratio. In several experiments the administration of the protein or the ribonucleic acid was repeated after 24 hours (Fig 1D). A full description of these protocols can be found here: dx.doi.org/10.17504/protocols.io.h7jb9kn

Western blot

Western blot analysis was performed on fully-confluent Cre-responsive cell lines harvested from 24-well plates 6 or 22 hours after the single administration of PTD-Cre (15 nM) or syn-mRNA-Cre (2.1 nM). The cells were lysed using RIPA buffer composed of 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0 [27]. Cell lysates (14 μg total protein per well) were mixed with 4x Laemmli loading buffer containing 8% SDS, 40% glycerol, 0.02% bromophenol blue, 250 mM Tris, and 20% 2-mercaptoethanol (all from Sigma-Aldrich), pH 6.8, heated at 95˚C for 3 min, and run on a 15% polyacrylamide gel and transferred to PVDF membranes (Merck Millipore) using a Pierce G2 electroblotter (Thermo Fisher Scientific). The membranes were blocked with 3% BSA (Sigma-Aldrich). Primary antibodies included a rabbit anti-T7 antibody (Abcam, Cambridge, UK) for detecting Cre recombinase (1:2000 dilution) and a mouse anti-beta-actin antibody (Sigma-Aldrich) as a loading control (1:7500 dilution). The secondary antibodies included goat anti-rabbit IgG-HRP (Merck Millipore) and rabbit anti-Mouse IgG-HRP (Thermo Fisher Scientific), each diluted 1:50000. Chemiluminescent SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) was used for detection. The signals were acquired using a G:BOX Chemi XR5 (Syngene, Cambridge, UK).

Fluorescence microscopy

Fluorescence microscopy was performed on Cre-responsive cell lines six days after a single administration of PTD-Cre (15 nM) or syn-mRNA-Cre (2.1 nM) to 10% confluent culture.

The cells were cultured on untreated glass coverslips in 48-well culture plates (Sigma-Aldrich) and then fixed with 4% formaldehyde (Polysciences, Warrington, PA). The cell nuclei were counterstained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) (Thermo Fisher Scientific). Stained cover slips were mounted on slides with Mowiol mounting medium. Cell samples were imaged using an EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific).

Flow cytometry

Cre-responsive cell lines at 10% confluence were treated with a single and double administration of PTD-Cre or syn-mRNA-Cre using the three above-mentioned concentrations. Six days later, the cells were detached from the flat bottoms of 48-well plates (area 0.95 cm^2) using 0.63% trypsin (both from Sigma-Aldrich). Single-cell suspensions were washed and stored at 4˚C in PBS buffer for up to 2 hours and analyzed using a BD LSRII analyzer (Becton Dickinson). A total of 5000–10000 events were counted for each sample. The respective untreated cells were used as negative controls for gating.

Data evaluation and statistics

All experiments were carried out independently in triplicate, and the results are expressed as the mean ± standard deviation (SD). GraphPad Prism 5 was used to construct asymmetrical (five-parameter) dose-response curves and to calculate two-tailed unpaired Student's *t*-tests. P-values <0.05 were considered statistically significant.

Results and discussion

Cre-responsive cell lines

Cre-responsive cell lines were created from the original cell lines PANC-1, BRIN-BD11 and HEK293 by the genomic insertion of the expression cassette shown in Fig 1A and specified in S1 Fig, followed by single-cell sorting to produce clonal populations. After the sorting, approximately 20–30 of the 96 wells contained RFP-positive cells, depending on the original cell lines. For each cell line, three clones were expanded and preserved. A single clone of each line was then used throughout the study. After expansion, the presence of the construct was verified using fluorescence microscopy (RFP positivity). Theoretically possible inadvertent GFP expression in the absence of Cre recombinase (leakage) was excluded by the absence of a green fluorescence signal in any of the clones (Fig 2).

Detection of active Cre recombinase delivery into cell nuclei

Correct folding of the PTD-Cre fusion protein was assured by the chaperone action of the Maltose-Binding protein [28], which was encoded by the expression vector. There are a variety of PTD sequences [29], but predicting the best one for a particular cell type is not possible. Our preliminary experiments using TAT3-GFP and TAT8-GFP constructs suggested that the latter penetrated into PANC-1 cells better, and it was therefore used throughout the study. Cre-responsive cells grown on glass coverslips were treated with a single administration of PTD-Cre1 (15 μM) or syn-mRNA-Cre (2.1 nM) for 24 hours. Successful delivery of a functional Cre protein into the cell nuclei was verified microscopically by detecting the Cre-mediated synthesis of GFP in the cytoplasm of individual cells. The GFP signal became visible three days after treatment, irrespective of the cell type (fl-PANC, fl-BRIN, fl-HEK) or the delivery method. The signal reached a maximum around day 6 and maintained an apparently unchanged level for another 7 days (data not shown). The number of positive cells clearly

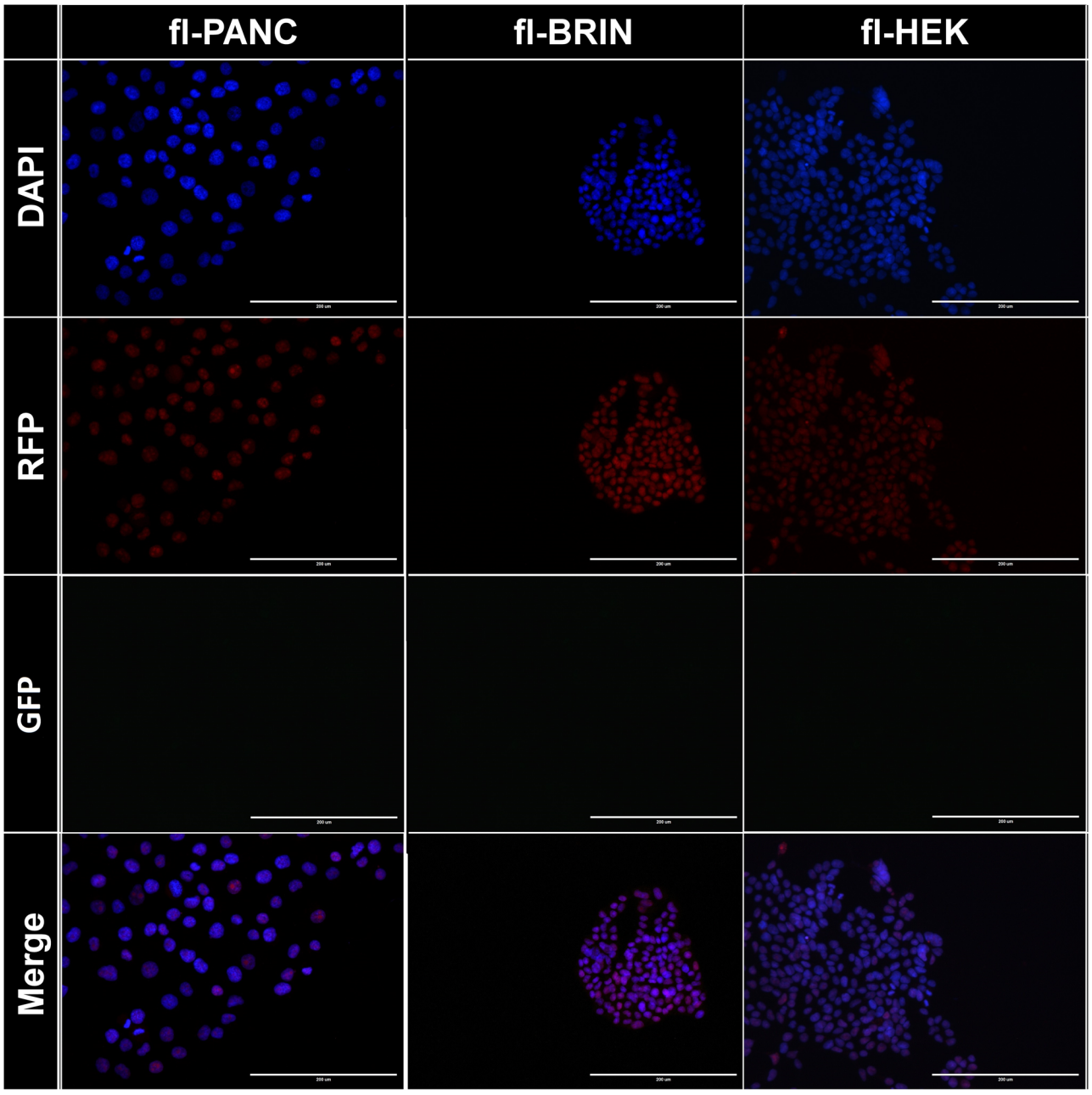

Fig 2. Verification of engineered clones after their expansion. No leaky GFP expression was observed.

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differed between the two delivery strategies and among the cell types. While approximately half of the syn-mRNA-treated cells from each cell line produced GFP, only a small fraction of cells was GFP-positive after treatment with PTD-Cre1. The best result quantified by flow cytometry was 0.123±0.067% (n = 3) of GFP^+ cells in fl-BRIN cells (data not shown). This failure occurred despite the presence of the nuclear localization sequence (NLS) in the PTD-Cre1 protein (S2 Fig). Spontaneous entry of Cre recombinase into the cell nucleus has been previously reported [30]. We modified our original construct accordingly by changing the domain

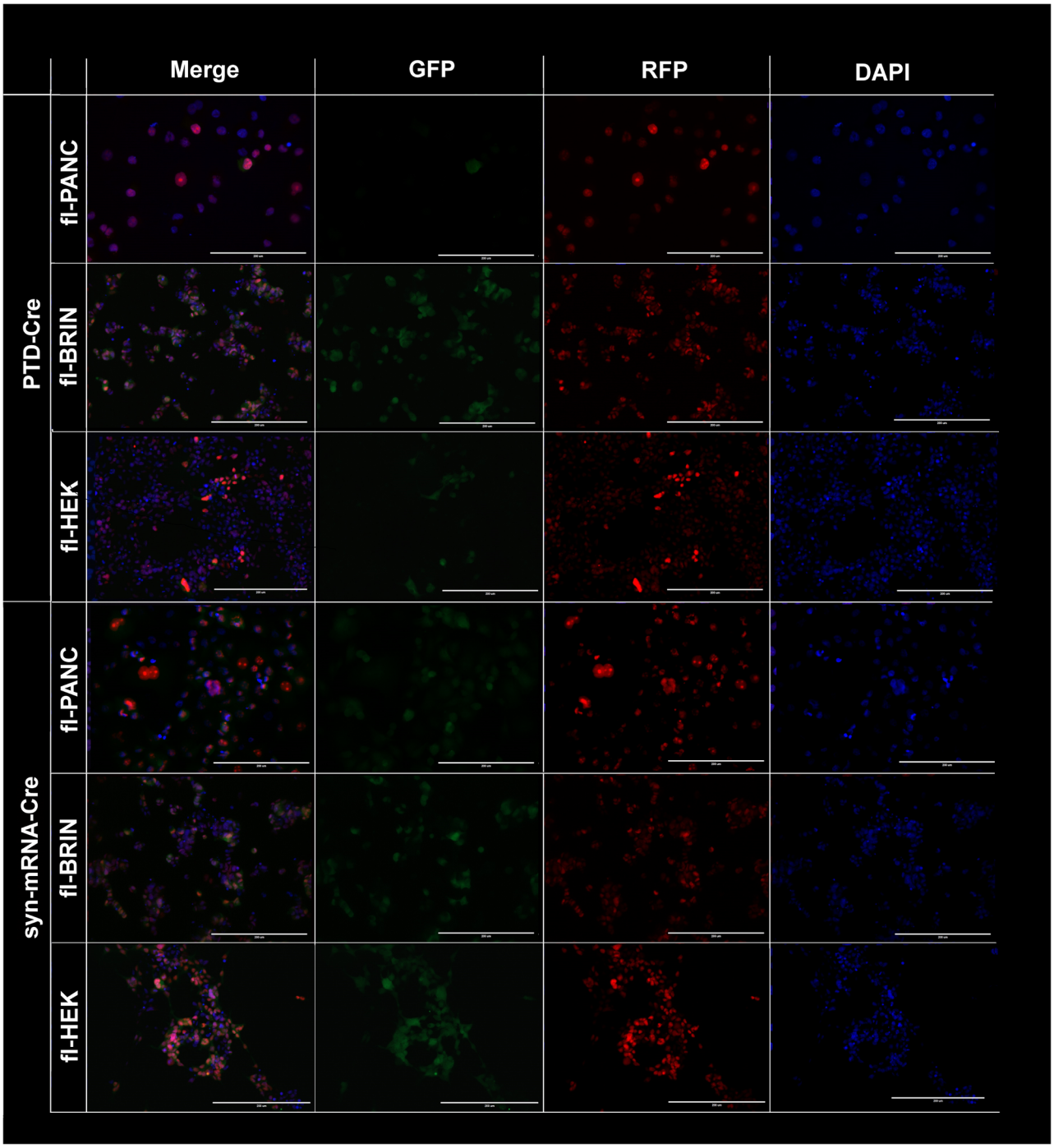

Fig 3. Detection of Cre recombinase activity in cell nuclei of the target cells after administration of PTD-Cre (15 μM) or syn-mRNA-Cre (2.1 nM). Bar 200 μm.

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order and removing the NLS (Fig 1B, S3 Fig). Using this PTD-Cre2 construct at the highest concentration (15 μM), the number of GFP-positive fl-BRIN cells increased, but there was no substantial change among the fl-PANC and fl-HEK cells (Fig 3). PTD-Cre2 was used in subsequent experiments.

Efficiency of Cre protein delivery into the cell nuclei

The Cre-responsive cells were grown on plastic dishes (area 0.95 cm^2) and treated with either PTD-Cre2 or syn-mRNA-Cre at low (10%) confluence to account for their expansion over a period of one week. Six days after the first administration (Fig 1D), the cells were harvested in a single-cell suspension and the GFP^+ cells were quantified using flow cytometry. Fig 4 depicts representative scatter plots obtained from each cell type after administration of either PTD-Cre2 (15 μM) or syn-mRNA-Cre (2.1 nM).

Direct comparison of the methods employing substances of different classes (protein vs. mRNA) was realized by calculating the individual doses relative to the maximum dose achievable for each substance. Protein precipitation limited the maximum protein concentration of PTD-Cre2 to 15 μM. It was practical to further increase the dose by repeated administration of this concentration (no toxicity was observed). The dose of syn-mRNA-Cre turned out to be limited by cell toxicity. After the double administration of 2.1 nM of syn-mRNA-Cre, dead cells appeared in the medium and the growth of the remaining attached cells was reduced. Double administration of lower concentrations had no visible toxic effects (data not shown). To prevent this innate cell toxicity, we used modified nucleotides [31, 32]. However, our syn-mRNA was not HPLC-purified. Such purification would reduce cytotoxic byproducts of the *in vitro* mRNA synthesis and might potentially allow for the use of even higher concentrations of syn mRNA-Cre [33].

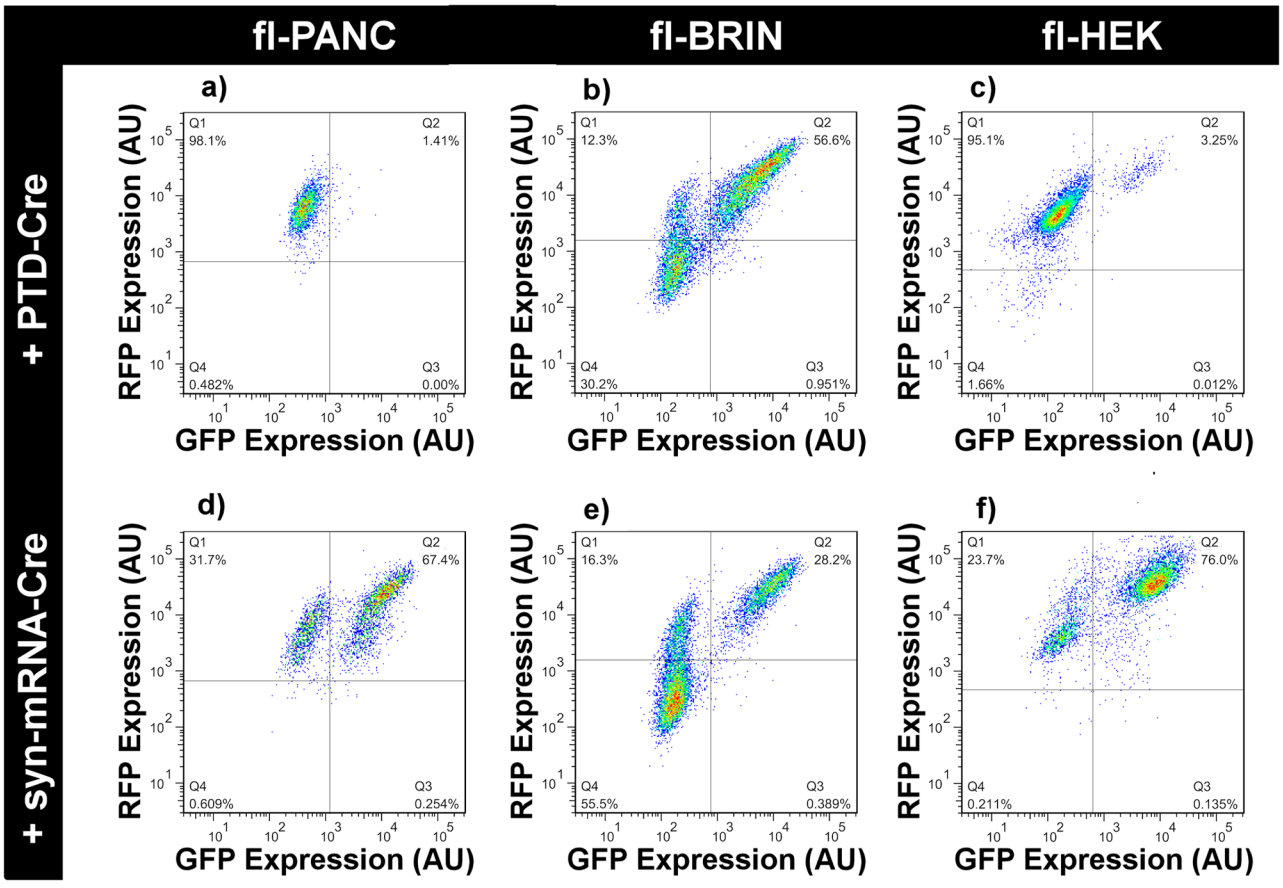

Fig 4. Flow cytometry scatter plots obtained on day 7 after single administration of 15 μM PTD-Cre (a-c) or 2.1 nM syn-mRNA-Cre (d-f) in fl-PANC (a,d), fl-BRIN (b,e), and fl-HEK (c,f) cells.

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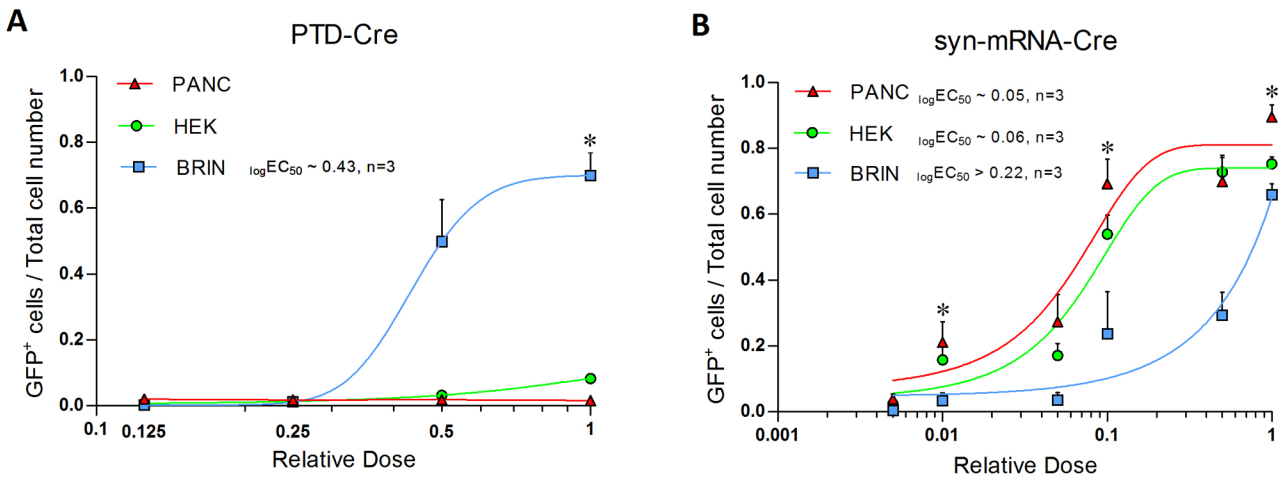

Fig 5. Frequency of GFP⁺ cells after the treatment of Cre-responsive cells with either PTD-Cre2 (A) or syn-mRNA-Cre (B). Analysis of flow cytometry data from three independent experiments. X-axis: relative dose on the logarithmic scale, (1 is the maximum dose); Asterisk, double administration; Y-axis: efficiency of the delivery of Cre recombinase into nuclei of fl-PANC (red triangles), fl-BRIN (blue squares), fl-HEK (green circles) cells; Error bars: mean±SD, n = 3; GraphPad was used to construct asymmetrical (five-parameter) dose-response curves and to calculate the approximate logEC50.

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The frequencies of successful delivery of Cre recombinase protein into cell nuclei by day seven after treatment with either PTD-Cre2 protein or syn-mRNA are summarized in Fig 5 for three independent experiments in the Cre-responsive cell lines. PTD-Cre2 mediated an appreciable delivery of an active Cre recombinase protein only in the nuclei of fl-BRIN cells (Fig 5A). Syn-mRNA-Cre was successful in all three cell types (Fig 5B). In the fl-PANC and fl-HEK cells (but not in fl-BRIN cells), the highest (toxic) dose was observed at the plateau of the dose-response curve (Fig 5B). Half maximum effective doses (EC50) were calculated as non-toxic doses at which the efficiencies of both delivery methods could be directly compared across the cell types. Table 1 demonstrates that PTD-Cre2 failed to deliver functional Cre recombinase into the nuclei of two out of three cell types, while the robustness of syn-mRNA-Cre was demonstrated by its substantial efficacy irrespective of cell type.

The two tested methods deliver the cargo protein inside the cells by different means. Therefore, differential robustness of the two methods could potentially be explained by different amounts of the Cre protein entering the treated cells. To determine the amounts of Cre protein that entered the cells, the Cre-responsive cells were treated with either PTD-Cre2 or syn-mRNA-Cre (single administration of the maximum concentrations) and harvested 6 or 22 hours later for western blot analysis. Fig 6 shows at each time point that the relative amount of Cre protein in cell homogenates was 4–19-fold higher in the syn-mRNA-treated cells than in the PTD-Cre2-treated cells. It is noteworthy, however, that the Cre protein in the PTD-treated fl-BRIN cells was 3–7 times lower than in the fl-HEK cells (p-values were 0.016 and 0.049 after 6 and 22 hr incubations, respectively), although the Cre-mediated recombination was

Table 1. Comparison of PTD-fusion and syn-mRNA. Half maximum effective doses (EC50) and half maximum efficiency in three cell types.

	Half Effective dose (EC50)		Half maximum efficiency	
	PTD-Cre (nmol/cm²)	syn-mRNA-Cre (pmol/cm²)	PTD-Cre (%)	syn-mRNA-Cre (%)
PANC	failed	0.047	failed	0.39
BRIN	2.71	0.205	0.38	0.24
HEK	failed	0.058	failed	0.37

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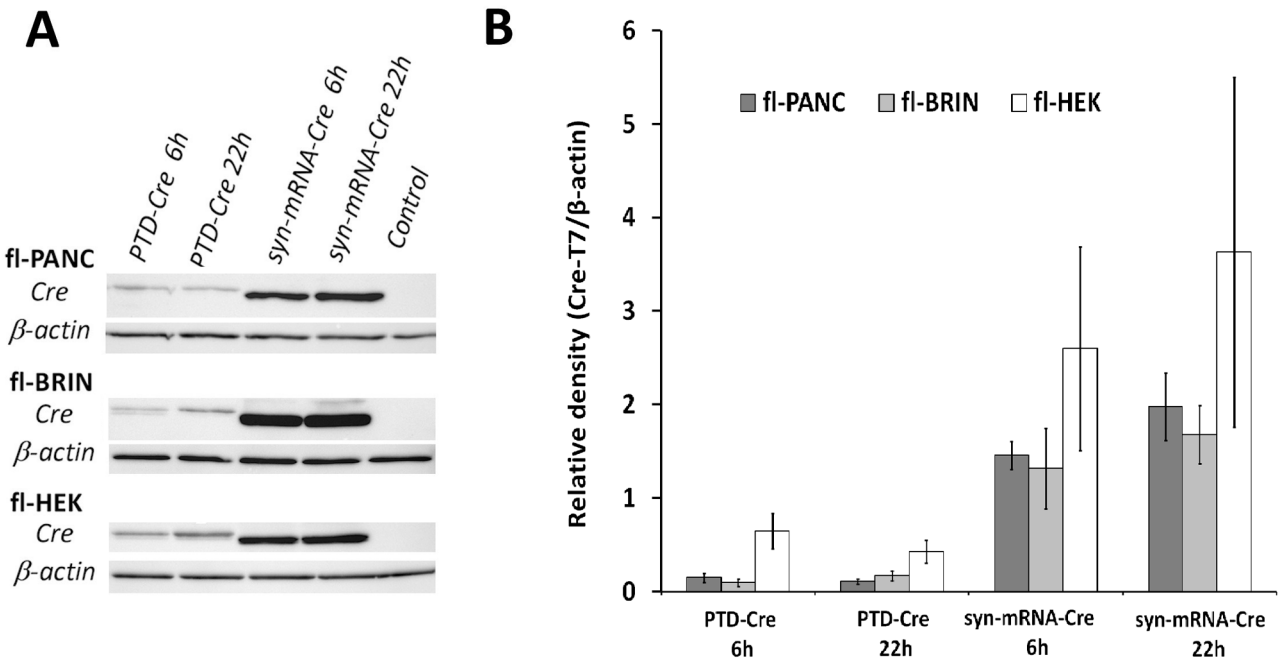

Fig 6. Quantification of intracellular Cre recombinase protein in three cell types at two time points after single administration of either 15 μM PTD-Cre or 2.1 nM syn-mRNA-Cre. (A) Western blot images are representative of three independent experiments. (B) Relative quantification of Cre recombinase protein, mean±SD, *n=3*.

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successful in the former and failed in the latter. Using the PTD-Cre in fl-HEK cells, we obtained results similar to those previously published [27]. Further clarification of the observation that the intracellular level of the cargo protein did not correspond with its nuclear effect is beyond the scope of this manuscript.

Workload

Starting from the transformed bacteria, the preparation of the PTD-Cre protein was labor-intensive (Materials and methods) and took two full working days, yielding a total of 2–3 mg of protein from 1 L of culture. The treatment of cells in one well (0.95 cm^2) with the highest concentration required 0.1 mg of protein. Starting with the ready-made DNA template, the preparation of syn-mRNA-Cre took up to 8 h, yielding approximately 60 μg of the syn-mRNA-Cre. The treatment of cells in one well (area 0.95 cm^2) with the highest concentration required 0.2 μg of synthetic syn-mRNA-Cre. The *in vitro* synthesis of a specific synthetic mRNA required less time and effort than the multistep preparation of a purified recombinant protein.

Conclusion

We conclude that in comparison to the PTD fusion-based protocol, the synthetic mRNA-based method is less cell type-dependent, less work-intensive, and more efficacious for protein delivery into cell nuclei. We recommend synthetic mRNA as a first-line approach, particularly when the cell type of interest has not been previously tested.

Supporting information

S1 Fig. Expression cassette, nucleotide sequence.
(DOCX)

S2 Fig. PTD-Cre1 nucleotide sequence.
(DOCX)

S3 Fig. PTD-Cre2 nucleotide sequence.
(DOCX)

S4 Fig. Template DNA sequence for syn-mRNA-Cre.
(DOCX)

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Supervision: Tomas Koblas.

Writing – original draft: Ivan Leontovyc.

Writing – review & editing: David Habart.

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ELSEVIER

The Effect of Epigenetic Factors on Differentiation of Pancreatic Progenitor Cells Into Insulin-Producing Cells

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ABSTRACT

Differentiation of pancreatic progenitors into insulin-producing β cells is regulated by various transcription factors. To be expressed the genes coding these transcription factors need to be in accessible DNA. Whether a particular gene is present in a form of active euchromatin structure with accessible DNA or in an inactive heterochromatin structure with inaccessible DNA is determined by various epigenetic modifications. We studied the effect of epigenetic modifiers on differentiation of human nonendocrine cells into insulin-producing cells with the aim to evaluate the effect of epigenetic modifications in that process. Within 3 days of cultivation nonendocrine cells form isletlike cell clusters (ILCCs) containing mainly cytokeratin-19-positive cells. After cultivation with epigenetic modifiers and further differentiation, the highest number of C-peptide-positive cells (10.3% $\pm$ 2.9%) as well as glucagon-positive cells (7.2% $\pm$ 2.8%) was observed in a sample supplemented with a combination of 5-Aza-2′-deoxycytidine modifiers, BIX01294 and MC1568. In response to glucose stimulation (5 vs 20 mmol/L) these ILCCs secreted increased amounts of C-peptide (0.45 vs 1.05 pmol C-peptide/μg DNA). Control samples treated without any epigenetic modifiers showed significantly lower numbers of C-peptide-positive cells (3.5% $\pm$ 1.6%). These results showed that a combination of epigenetic modifiers 5-Aza-2′-deoxycytidine (BIX01294 and MC1568) significantly improved reproducible differentiation of nonendocrine pancreatic cells into insulin-producing cells.

THE APPLICATION OF insulin-producing tissue derived from alternative sources is a promising idea to treat diabetes mellitus. However, the efficiency of differentiation of various cell sources into insulin-producing cells is still relatively low despite progress in differentiation protocols. One of the key obstacles in this goal could be the DNA structure of genes coding key proteins involved in differentiation and function of mature β cells.[1] The expression of genes requires a less compact, accessible DNA in the form of an active euchromatin structure. In contrast, genes that are present in condensed DNA structures called *heterochromatin* are silenced and inactive. The condensation of chromatin is regulated by DNA methylation as well as by various modifications of histone proteins that encircle DNA. Generally, the modifications that determine the state of chromatin structure are termed *epigenetic modifications.* Recently published studies have shown that the epigenetic modifications have significant effects on the differentiation of pancreatic endocrine cells. Treatment of fetal pancreata with Trichostatin A (TSA), a nonspecific inhibitor of histone deacetylases, increased the number of differentiated endocrine cells including β cells.[2] Similar results were achieved with the application of 5-Aza-2′-deoxycytidine (5Aza), an inhibitor of DNA methyl transferase. 5Aza stimulated the expression of transcription factors involved in the differentiation of pancreatic endocrine cells and improved the differentiation of a ductal cell line into α and δ cells.[3] Therefore, we decided to evaluate the effect of various epigenetic modifiers on the differentiation of nonendocrine pancreatic cells into insulin-producing β cells.

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METHODS

Tissue Preparation

Human nonendocrine pancreatic cells were obtained from the pancreatic acinar tissue remaining after islet isolation. The isolation and transplantation program for human pancreatic islets was approved by our Ethics Committee. Human islets and pancreatic tissue were isolated according to the previously described methods.[4] Briefly, islets were isolated from pancreata obtained from five donors of mean age 46 ± 20 years. The pancreatic duct was perfused with a cold enzyme mixture containing Collagenase NB 1 Premium Grade and Neutral Protease NB (Serva, Heidelberg, Germany). Tissue was then transferred to a modified Ricordi chamber for separation by gentle mechanical agitation and enzymatic digestion at 37°C. Islets were purified with continuous gradients of Biocoll (Biochrom, Berlin, Germany) in an apheresis system Code model 2991 (Gambro, Prague, Czech Republic). The densities of the continuous gradient ranged from 1.065 to 1.092 g/mL. During centrifugation, islets migrated to the interface between 1.070 and 1.080 g/mL. The remaining cellular suspension from the denser layer was pooled and further digested in Accutase solution (Sigma-Aldrich, Steinheim, Germany) for 20 minutes at 37°C. The single-cell suspension obtained after a filtration through 11-μm cell strainer was purified with a Biocoll continuous gradient in an apheresis system. The cell suspensions obtained from 1.050 to 1.080 g/mL interfaces were pooled and washed in Hanks solution (Sigma-Aldrich) for further processing.

Cell Culture Studies

Nonendocrine cells were cultured for 4 days (stage 1) in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% KnockOut serum replacement, 1% insulin-transferrin-selenium, 25 U/mL penicillin, 25 μg/mL streptomycin, 1 mmol/L L-glutamine, 1% nonessential amino acids, 0.1 mmol+L 2- mercaptoethanol (all from Invitrogen, Paisley, UK), 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF) (both from Peprotech, Rocky Hill, NJ, USA) and conditioned medium derived from neonatal fibroblasts cell line Hs68 (LGC Promochem, Teddington, UK). During stage 1, cells were divided into 13 groups for supplementation with epigenetic modifiers according to Table 1 The epigenetic modifiers included: 1 μmol/L 5Aza, a DNA methyltransferase inhibitor; 1 μmol/L Scriptaid, an inhibitor of class I + II histone deacetylases; 500 mmol/L sodium butyrate, an inhibitor of class I + II histone deacetylases; 5 μmol/L MC1568, an inhibitor of class II histone deacetylases; and 2 μmol/L BIX01294, an inhibitor of G9a histone H3K9 methyltransferase (all from Sigma-Aldrich). Afterward, cells were cultured for 3 days (stage 2) in CMRL medium containing 5% FCS, 10 μmol/L SP600125, 10 μmol/L SB 216763, 10 μmol/L forskolin, 5 μg/mL fibronectin, 10 mmol/L nicotinamide, 40 ng/mL Exendin-4 (all from Sigma-Aldrich), and 100 ng/mL IGF (Peprotech). Within the first 3 days cells formed clusters, further referred to as isletlike cell clusters (ILCC).

Reverse Transcriptase Polymerase Chain Reaction

Total RNA from approximatelly 10^6 cells was isolated using the Rneasy Plus Mini Kit (Qiagen, Hilden, Germany) for treatment with DNAse using the RNase-Free DNase Set (Qiagen). Isolated RNA (1 μg) was reverse transcribed with an Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. cDNA was amplified using HotStarTaq Master Mix Kit (Qiagen). Total RNA from the islet fraction was used as the positive control. Gene-specific primer pairs, annealing temperatures, and product size were performed as previously described.[5] Separated polymerase chain reaction (PCR) products were visualized on 2% agarose gel using ethidium bromide.

Immunocytochemistry

ILCC washed with phosphate-buffered saline (PBS) were fixed for 60 minutes in Bouin's solution (Sigma-Aldrich), rinsed with PBS, suspended in a 2% agarose-PBS solution, and centrifuged at 100*g* for 3 minutes to form compact pellets. After overnight submersion in 30% sucrose, ILCC were embedded in optimal cutting temperature mounting medium TissueTek (Bayer Corp, Pittsburgh, Pa, USA), frozen in liquid nitrogen, and stored at −80°C.

After several washes in PBS, 8-μm sections of frozen ILCC were incubated in blocking solution containing 10% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, Pa, USA) in 0.2% Triton X-100, 0.1 mol/L glycine (Sigma-Aldrich), and PBS for 1 hour at room temperature to prevent nonspecific binding. Incubation with primary antibodies in appropriate dilution was performed in a blocking solution for 1 hour at 37°C. The following primary antibodies were used at a 1:100 dilution: mouse anti-cytokeratin 19, mouse anti-C-peptide (both from Exbio, Czech Republic) and rabbit anti-glucagon (Cell Signaling, Danvers, Mass, USA). After intensive washing with PBS, sections were incubated with the specific secondary antibody diluted in the blocking solution for 1 hour at 37°C. The secondary antibodies were Alexa Fluor 555 donkey anti-mouse immunoglobulin G (IgG) and Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen). 4,6-Diamidino-2-phenylindole (Sigma-Aldrich) at a concentration 5 μg/mL was used to label the nuclei for 10 minutes at 37°C. After rinsing with PBS, sections mounted with antifade solution were examined using a fluorescence microscope Olympus BX41 (Olympus, Tokyo, Japan).

Table 1. Combinations of Epigenetic Modifiers Used in the Study

	Group												
	1	2	3	4	5	6	7	8	9	10	11	12	13
5Aza	A	A	A	A	A	A							
Sodium butyrate			SB			SB			SB			SB	
Scriptaid	S			S			S			S			
MC1568		M			M			M			M		
BIX01294	B	B	B				B	B	B				

Combinations of epigenetic modifiers used in the study. Cell samples were divided into 12 groups with various combinations of 5Aza (A), sodium butyrate (SB), Scriptaid (S), MC1568 (M), and BIX01294 (B). Control sample was treated without any of epigenetic modifiers. 5Aza, 5-Aza-deoxycitidine.

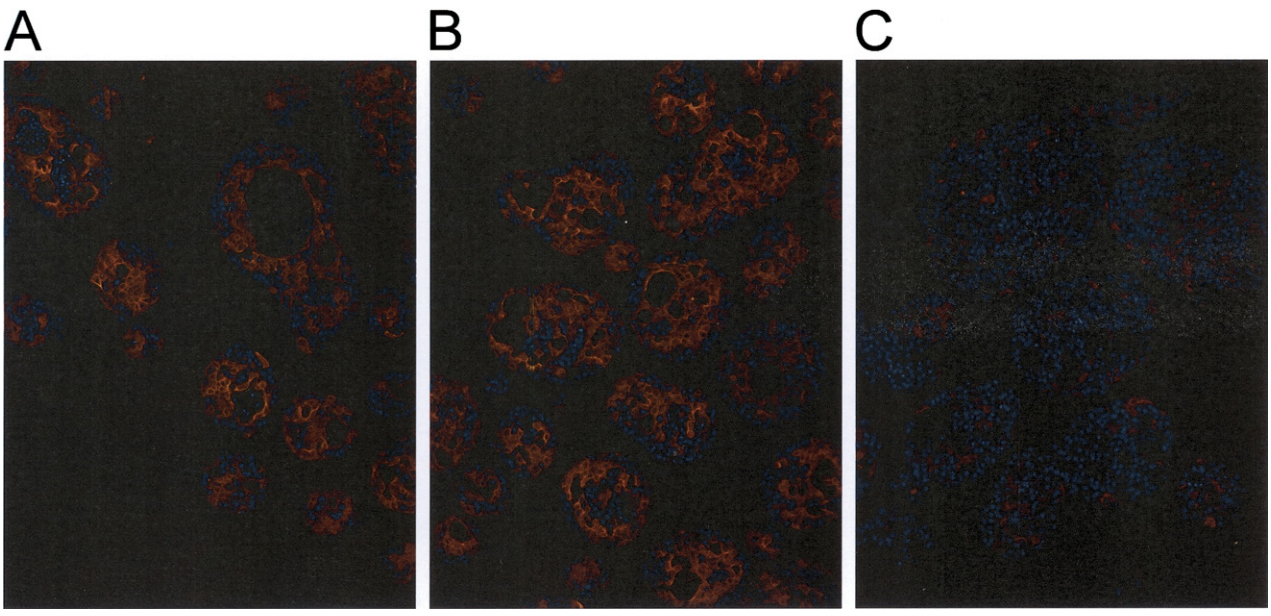


Fig 1. Cytokeratin-19 immunofluorescence staining of isletlike cell clusters (ILCCs). Immunoflourescence staining of cytokeratin-19 (orange): ILCCs treated with a combination of 5-Aza-2′-deoxycitidine (5Aza), MC1568, and BIX01294 **(A)**, control ILCCs treated without any of the epigenetic modifiers **(B)**, ILCCs treated with a combination of 5Aza and MC1568 **(C)**. ,6-Diamidino-2-phenylindole (blue) stain was performed as counter stain (magnification 100×).

C-Peptide Cell Content and Glucose-Stimulated Secretion

C-peptide release was measured by incubating 100 ILCC in 1 mL of Krebs-Ringer solution containing 5 mmol/L glucose for 1 hour and then in 20 mmol/L glucose solution for another hour. Cells were lysed in RIPA buffer (Sigma-Aldrich) and human C-peptide determined using a C-peptide immunoradiometric kit (Beckman Coulter, Fullerton, Calif, USA) according to the manufacturer's instructions.

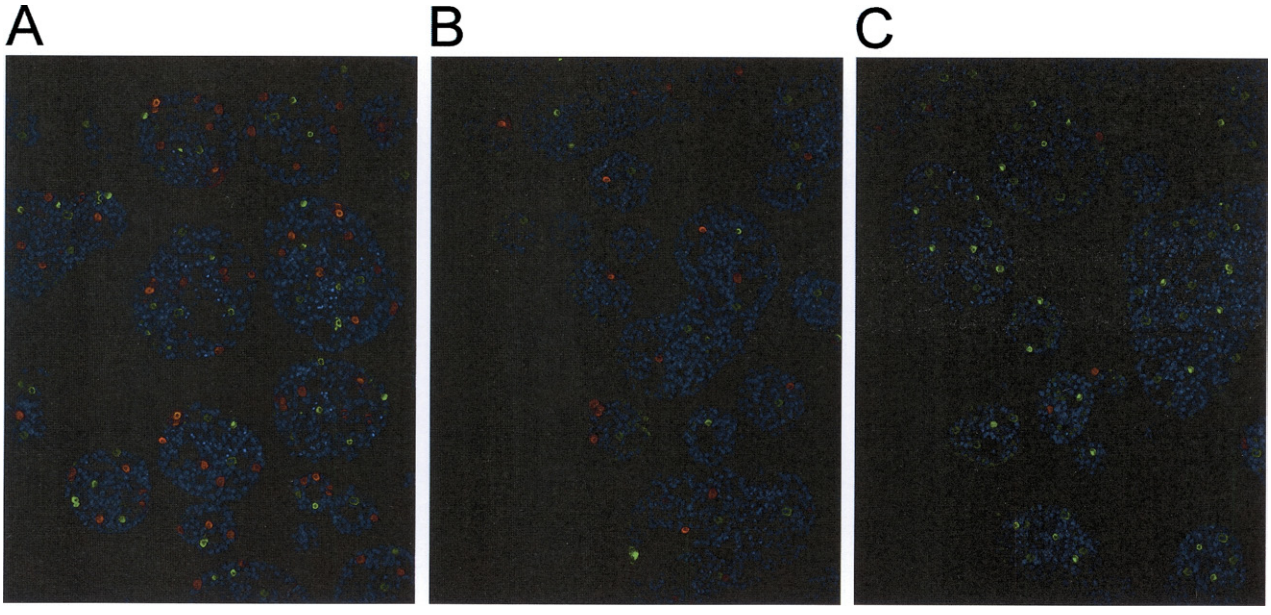
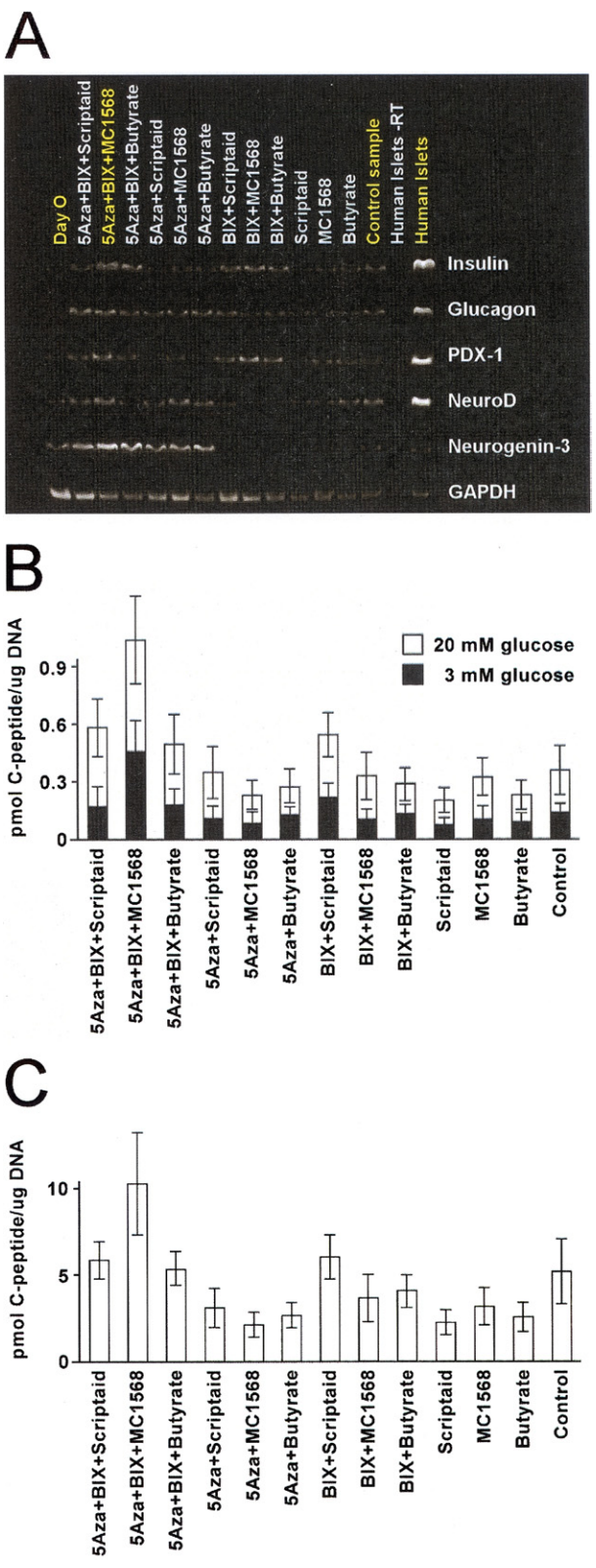


Fig 2. C-peptide and glucagon immunofluorescence staining of isletlike cell clusters (ILCCs). Immunoflourescence staining of C-peptide (orange) and glucagon (green): ILCCs treated with a combination of 5-Aza-2′-deoxycitidine (5Aza), MC1568, and BIX01294 **(A)**, control ILCCs treated without any of the epigenetic modifiers **(B)**, ILCCs treated with a combination of 5Aza and MC1568 **(C)**. 4,6-Diamidino-2-phenylindole (blue) stain was performed as counter stain (magnification 100×).



Statistical Analysis

Statistical analysis was performed using Student *t* test. All data are presented as mean values ± standard deviations. P values $< .05$ were deemed to be significant.

RESULTS

Single-cell suspensions of nonendocrine pancreatic cells formed clusters (ILCC) resembling the islets of Langerhans within 3 days of culture in serum-free neonatal fibroblast-conditioned medium. ILCC were formed mainly by cytokeratin-19-positive cells with the exception of 5Aza- and MC1568-treated ILCC that contained only rare cytokeratin-19-positive cells after 7 days of cultivation (Fig 1). The highest number of C-peptide-positive cells (10.3% ± 2.9%) was observed in samples treated with a combination of 5Aza, BIX01294, and MC1568. There was also a high number of glucagon-positive cells (7.2% ± 2.8%) in samples treated with this combination of epigenetic modifiers. Control samples treated without any epigenetic modifier contained only 3.5% ± 1.6% C-peptide-positive cells. Samples treated with 5Aza + MC1568 displayed the smallest number of C-peptide-positive cells (1.8% ± 0.9%), while the number of glucagon-positive cells (8.1% ± 1.4%) was the greatest in all samples (Fig 2). Analysis of C-peptide protein content agreed with the results from the immunofluorescence staining. The highest C-peptide content was detected in samples treated with 5Aza, BIX01294, and MC1568(10.2 ± 3.2 pmol C-peptide/μg DNA), while the C-peptide content in control samples was significantly lower (5.1 ± 2.2 pmol C-peptide/μg DNA; Fig 3). Insulin secretory capacity of differentiated ILCCs was confirmed by glucose-stimulated C-peptide secretion. In response to glucose stimulation (3 vs 20 mmol/L), ILCCs treated with 5Aza, BIX01294, and MC1568 secreted 0.45 versus 1.05 pmol C-peptide/μg DNA (Fig 3).

Results from immunofluorescence staining were also consistent with results from reverse transcriptase PCR analysis (Fig 3). The highest insulin gene expression was observed among ILCCs treated with 5Aza, BIX01294, and MC1568. The expression of the glucagon gene was also high in samples treated with 5Aza, BIX01294, and MC1568 compared with most other samples.

Treatment of pancreatic cells with epigenetic modifiers also affected the expression of key transcription factors of endocrine cell differentiation. The expression of pancreatic and duodenal homeobox 1 (PDX-1) transcription factor was significantly higher in samples treated with BIX01294,

Fig 3. Gene expression and C-peptide secretion capacity and content of differentiated isletlike cell clusters (ILCCs). Reverse transcriptase polymerase chain reaction analysis of gene expression in treated isletlike cell clusters (ILCCs). A negative control (without reverse transcription) and a positive control (human islets). **(A)**. Glucose stimulated C-peptide secretion of differentiated ILCCs as determined by IRMA **(B)**. C-peptide content of differentiated ILCCs as determined by IRMA **(C)**.

while treatment with 5Aza stimulated the expression of neurogenin-3 transcription factor.

DISCUSSION

Our knowledge about basic principles of epigenetic modifications that significantly affect cell differentiation has improved during the last decade. However, there is still little known about epigenetic modifications that regulate differentiation of pancreatic β cells.

Our results showed that epigenetic factors significantly affect differentiation of human nonendocrine pancreatic cells into insulin-producing cells. Our findings are consistent with a recent study that showed a positive effect of the histone deacetylase inhibitor TSA on differentiation of mouse fetal pancreatic cells into β cells.[2] In another study, treatment of a human ductal cell line with the DNA methyltransferase inhibitor 5Aza stimulated differentiation into α and δ cells as well as the expression of neurogenin-3, an important transcription factor for differentiation of pancreatic endocrine cells.[3]

Therefore, we decided to evaluate the effect of various combinations of epigenetic modifiers that have already been shown to be effective in the differentiation of pancreatic endocrine cells as well as of other cell types. We observed that the combination of 5Aza (DNA methyltransferase inhibitor), MC1568 (specific inhibitor of class II histone deacetylases), and BIX01294 (specific inhibitor of G9a histone H3K9 methyl transferase) improved differentiation of human pancreatic nonendocrine cells. The positive effects of that combination of epigenetic modifiers may be due to complex changes in DNA methylation and histone modifications. While 5Aza decreases methylation of DNA, MC1568 blocks enzymes responsible for deacetylation of histones and BIX01294 inhibits methylation of lysine 9 on histone 3. All of these modifiers are supposed to stimulate a change of DNA structure into the euchromatin state necessary for gene expression. Indeed, in our study various combinations of epigenetic modifiers stimulated differentiation into endocrine cells; however, with significantly different results. The combination of 5Aza and MC1568 deteriorated differentiation into β cells, while it increased differentiation into α cells compared with control samples. In contrast, the combination of 5Aza, MC1568, and BIX01294 significantly stimulated differentiation into β cells. Therefore, addition of BIX01294 played a key role in directing differentiation of pancreatic cells into the β-cell phenotype.

However, we were not able to achieve the same level of differentiation into β-cell phenotype with the combination of only BIX01294 and MC1568. Therefore, the addition of 5Aza seemed to be beneficial for differentiation of ILCCs cells into the β-cell phenotype. In our study, we confirmed previous results that 5Aza stimulates the expression of neurogenin-3, a key transcription factor for differentiation of pancreatic endocrine cells. While 5AZA could trigger the endocrine differentiation process by stimulating the expression of neurogenin-3, the addition of BIX01294 further stimulated differentiation into a β-cell phenotype by inducing PDX-1 expression.

In addition to their effects on endocrine cell differentiation, we also observed actions of epigenetic modifiers on the number of cytokeratin-19-positive cells. For example, treatment with 5Aza and MC1568 significantly decreased the number of cytokeratin-19-positive cells in comparison with control samples. However, since epigenetic modifiers affected the structure of the whole genome of DNA and not only a small group of genes, effects on other cell types are not surprising.

In conclusion, our study demonstrated that application of various epigenetic modifiers stimulated differentiation of human nonendocrine pancreatic elements into insulin-producing cells with expression of key transcription factors of endocrine cell differentiation. The combination of these modifiers with other agents that stimulate differentiation of pancreatic nonendocrine cells may improve the yield of differentiated beta cells allowing this potential source of insulin-producing tissue to join the clinical treatment of diabetes.

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Reprogramming of Pancreatic Exocrine Cells AR42J Into Insulin-producing Cells Using mRNAs for Pdx1, Ngn3, and MafA Transcription Factors

Tomas Koblas[1], Ivan Leontovyc[1], Sarka Loukotova[1], Lucie Kosinova[1] and Frantisek Saudek[2]

Direct reprogramming of pancreatic nonendocrine cells into insulin-producing β-cells represents a promising approach for the treatment of insulin-dependent diabetes. However, its clinical application is limited by the potential for insertional mutagenesis associated with the viral vectors currently used for cell reprogramming. With the aim of developing a nonintegrative reprogramming strategy for derivation of insulin-producing cells, here, we evaluated a new approach utilizing synthetic messenger RNAs encoding reprogramming transcription factors. Administration of synthetic mRNAs encoding three key transcription regulators of β-cell differentiation—Pdx1, Neurogenin3, and MafA—efficiently reprogrammed the pancreatic exocrine cells into insulin-producing cells. In addition to the insulin genes expression, the synthetic mRNAs also induced the expressions of genes important for proper pancreatic β-cell function, including *Sur1*, *Kir6.2*, *Pcsk1*, and *Pcsk2*. Pretreating cells with the chromatin-modifying agent 5-Aza-2′-deoxycytidine further enhanced reprogramming efficiency, increasing the proportion of insulin-producing cells from 3.5 ± 0.9 to 14.3 ± 1.9% (*n* = 4). Moreover, 5-Aza-2′-deoxycytidine pretreatment enabled the reprogrammed cells to respond to glucose challenge with increased insulin secretion. In conclusion, our results support that the reprogramming of pancreatic exocrine cells into insulin-producing cells, induced by synthetic mRNAs encoding pancreatic transcription factors, represents a promising approach for cell-based diabetes therapy.

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Subject Category: Gene Insertion, Deletion & Modification

Introduction

Reports of whole pancreas transplantations and transplantations of isolated pancreatic islets demonstrate that replacement of insulin-producing tissue can potentially cure insulin-dependent diabetes.[1] However, use of this therapeutic approach is limited by a lack of suitable organ donors and the need for permanent immunosuppression. Thus, there remains a need for a safe and plentiful source of insulin-producing cells. One of the most promising methods is the differentiation of embryonic stem cells and induced pluripotent stem cells into insulin-producing cells, mainly due to its high efficiency and the high quality of derived cells.[2,3] However, the clinical application of this method may be limited by the potential risk of transformation into malignant cells.[4,5]

Cell reprogramming has recently emerged as another promising means of generating insulin-producing cells. A terminally differentiated cell can be directly reprogrammed into the desired cell type via temporal expression of transcription factors that activate the transdifferentiation program. Specific transcription factor combinations can induce reprogramming of fibroblasts into neurons,[6] cardiomyocytes,[7] hepatocytes,[8] and induced pluripotent stem cells.[9,10] Similarly, pancreatic exocrine cells and liver bile duct epithelial cells can be transdifferentiated into insulin-producing cells through induced expression of the transcription factors Pdx1, Neurogenin3, and MafA, which participate in the natural differentiation of pancreatic β-cells.[11–13] Insulin-producing cells derived from exocrine or liver cells by *in vivo* reprogramming reportedly normalize blood glucose levels in diabetic mice, demonstrating their therapeutic potential.[14,15]

Viral vectors are often used to introduce specific transcription factors into cells for reprogramming. However, highly efficient lentiviral and retroviral vectors can lead to the integration of viral DNA sequences into chromosomal DNA, potentially causing tumorigenic transformation.[16,17] Likewise, adenoviral vectors that are considered to be nonintegrating, tend to integrate viral DNA into the host genome, although at a low frequency.[18,19] Therefore, a truly integration-free reprogramming method could substantially improve the safety of the derived cells for eventual clinical application. Several integration-free techniques, utilizing episomal plasmids,[20] recombinant proteins,[21] Sendai RNA virus,[22] miRNA,[23] and synthetic mRNA have been recently reported.[24] While each of these methods has both advantages and disadvantages, the most efficient method appears to be cell reprogramming using synthetic mRNAs encoding reprogramming factors.[25]

The present study aimed to develop a safe and integration-free method of reprogramming pancreatic exocrine cells into insulin-producing cells. For this purpose, we chose the AR42J cell line. AR42J is a rat pancreatic exocrine cell line derived from a chemically induced pancreatic tumor.[26] It has been previously used as a model cell line for

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Keywords: β-cells; diabetes; insulin-producing cells; MafA; modified mRNA; Neurogenin 3; Pdx1; reprogramming; synthetic mRNA; transdifferentiation
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the analysis of pancreatic exocrine cells transdifferentiation into insulin-producing cells induced by adenoviral vectors encoding Pdx1, Neurogenin3, and MafA transcription factors.[11,12] Unlike primary exocrine cells, AR42J cells possess both exocrine and neuroendocrine properties as evidenced by the expression of the neuroendocrine-specific vesicle proteins synaptophysin and S.V.2 (ref. 27). Mixed exocrine-neuroendocrine character of these cells is further evidenced by the considerable amounts of neurotransmitters glycine, glutamine, and γ-aminobutyric acid. However, AR42J cells do not express any of the islet hormones under the standard culture conditions.[28] Moreover, AR42J cells have a stable phenotype upon *in vitro* culture and do not tend to undergo a ductal transdifferentiation under adherent culture conditions, like primary pancreatic exocrine cells do.[11]

Reprogramming factors were delivered into the exocrine cells in a form of synthetic mRNAs encoding the pancreatic transcription factors Pdx1, Neurogenin3, and MafA. Temporary expression of these reprogramming factors activated transdifferentiation of pancreatic exocrine cells into insulin-producing cells that expressed characteristic pancreatic β-cell markers and could process proinsulin into mature insulin and its byproduct C-peptide. The reprogrammed cells responded to glucose stimulation with limited insulin secretion, similar to that of immature β-cells.[29] Our results represent the first proof that it is feasible to generate insulin-producing cells through the transdifferentiation of exocrine pancreatic cells using an integration-free protocol based on synthetic mRNAs.

Results

Induced expression of reprogramming factors upon intracellular delivery of synthetic modified mRNAs

Cell reprogramming relies on ectopic expression of reprogramming transcription factors. Therefore, we first evaluated the efficiencies of transfection of each individual synthetic mRNA and expression of the encoded pancreatic transcription factors Pdx1, Neurogenin3, and MafA by the pancreatic exocrine cell line AR42J. Immunofluorescence staining revealed that transcription factor expressions were dose-dependent, with maximal expression rates achieved at a concentration exceeding 1–2 µg mRNA/ml media 20 hours post-transfection (**Figure 1b**,**c**). At a dose of 1 µg mRNA/ml media, Pdx1 was efficiently expressed by most cells (72.1 ± 7.4%, n = 5) while the expression rates of MafA (66.7 ± 11.3%, n = 5) and Neurogenin3 (36.9 ± 10.9%, n = 5) were lower and more variable as revealed by immunofluorescence staining (**Figure 1b**,**c**). Even at a higher mRNA concentration of 2 µg/ml media, variable expression was still detected, mainly for Neurogenin3 and MafA (**Figure 1b**,**c**).

Since mRNA stability is one of the key parameters determining the gene expression rate, we also evaluated the post-transfection stability of the synthetic mRNAs. Within 4 hours, synthetic mRNA was detected in cells. The highest level of synthetic mRNA was detected between 12–16 hours post-transfection. The level of synthetic mRNA in cells substantially decreased by 24 hours post-transfection (**Figure 2a**), although some synthetic mRNA was detected even at 36 hours post-transfection.

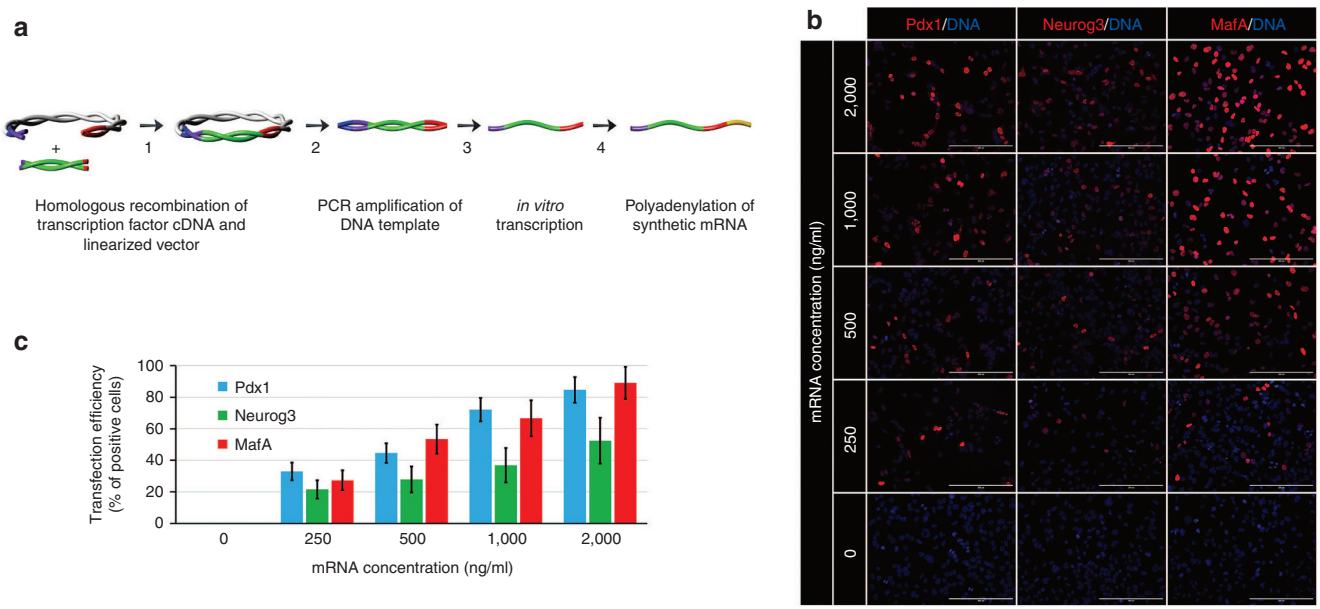

Figure 1 Scheme of DNA template construct production, *in vitro* transcription, and determination of efficiencies of transfection and expressions of synthetic mRNAs of the transcription factors Pdx1, Neurogenin3, and MafA by the pancreatic exocrine cell line AR42J. (**a**) Production of DNA template constructs and subsequent mRNA synthesis: (1) homologous recombination of transcription factor cDNA and linearized vector containing the T7 promoter, the 5′UTR (untranslated region) of the rat β-globin gene, and the 3′UTR of the human β-globin gene; (2) PCR amplification of DNA template; (3) *in vitro* transcription; and (4) polyadenylation of synthetic mRNA. (**b**, **c**) Dose-dependent expressions of Pdx1, Neurogenin3, and MafA upon transfection of AR42J cells with synthetic mRNAs at doses of 0, 250, 500, 1,000, and 2,000 ng/ml media as determined by immunofluorescence staining 20 hours post-transfection. Cell nuclei are stained blue with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DNA). Scale bars = 200 µm. Values are shown as mean ± standard deviation (n = 5).

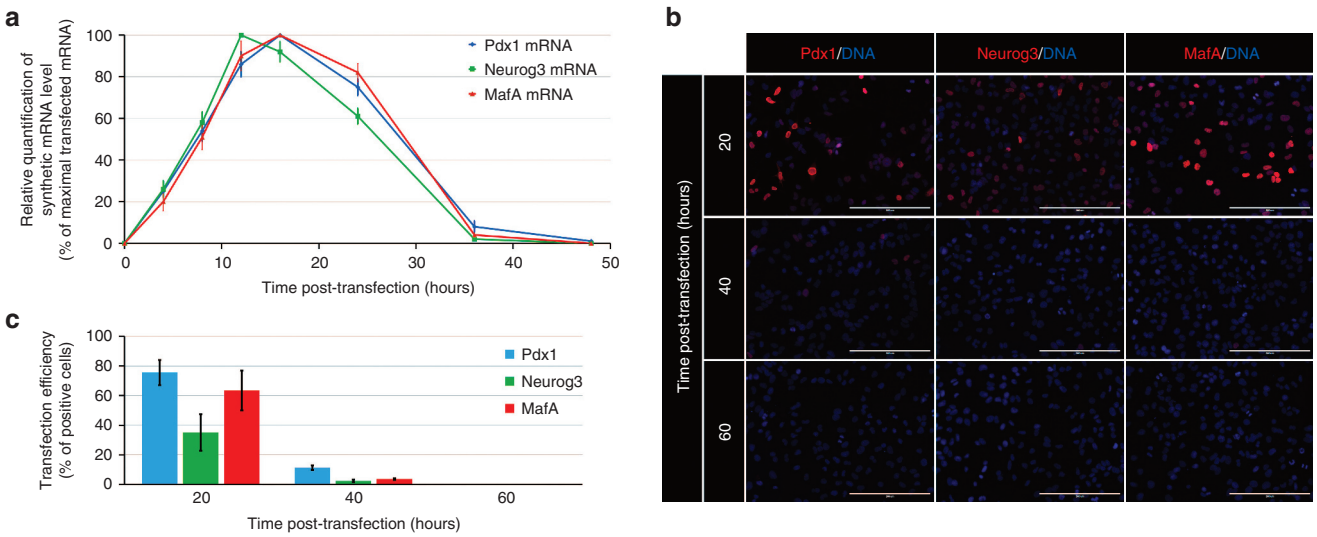

Figure 2 Stability of synthetic mRNAs of the transcription factors Pdx1, Neurogenin3, and MafA. (**a**) Stability of synthetic mRNAs for Pdx1, Neurogenin3, and MafA upon their transfection into AR42J cells as revealed by quantitative reverse transcription polymerase chain reaction ($n = 3$). (**b**, **c**) Immunofluorescence staining results showing the stability of Pdx1, Neurogenin3, and MafA at 20, 40, and 60 hours after transfection of AR42J cells with the corresponding synthetic mRNAs at a dose of 1 µg mRNA/ml media. Cell nuclei (DNA) are stained blue by 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride. Scale bars = 200 µm. Values are shown as mean ± standard deviation ($n = 5$).

At the protein level, expression of the encoded pancreatic transcription factors was most intense at 20 hours after transfection of cells with synthetic mRNAs at a dose of 1 µg mRNA/ml media (**Figure 2b**,**c**). Positive staining was detected even 40 hours post-transfection, although the staining intensity and the number of positive cells significantly declined. All positive cells disappeared within 60 hours post-transfection.

Simultaneous coexpression of reprogramming transcription factors upon intracellular delivery of synthetic modified mRNAs

Efficient cell reprogramming requires simultaneous expression of transcription factors. Therefore, we evaluated the coexpression of the reprogramming transcription factors upon simultaneous transfection of cells with all three synthetic mRNAs (Pdx1, Neurogenin3, and MafA) at a dose of 500 ng of each mRNA/ml media (**Figure 3a**). Transcription factor coexpression was mainly limited by the expression rates of Neurogenin3 and MafA, since Pdx1 was expressed by most of the Neurogenin3- and MafA-positive cells. The rates of double-positive cells were 16.1 ± 3.8% ($n = 4$) for Pdx1 and MafA, 13.3 ± 2.8% ($n = 4$) for Pdx1 and Neurogenin3, and 11.7 ± 3.6% ($n = 4$) for MafA and Neurogenin3 (**Figure 3b**).

Supplementation with vaccinia virus-derived type I interferon receptor B18R prevents cell death during repeated transfection of synthetic modified mRNAs

Efficient cell reprogramming also requires that transcription factor expression continue over a sufficient time period. Therefore, synthetic mRNA was delivered in the form of lipid complexes, allowing repeated transfection. However, repeated daily transfection with synthetic mRNAs at a dose exceeding 1 µg/ml led to the induction of apoptosis and substantial cell loss over the 3-day period (**Supplementary Figure S1**). This may have been due to activation of the cellular innate immune response, which serves as an antiviral defense mechanism against DNA and RNA viruses[30] and is characterized by inflammatory cytokine production, protein synthesis inhibition, and apoptosis induction.[31] Innate immune response activation by exogenous mRNA can be limited by incorporating modified nucleotide bases into the synthetic mRNA[24,30,32] and by dephosphorylation of 5′ triphosphates via phosphatase treatment.[24,33] However, using the modified nucleotides pseudouridine and 5-methylcytidine in our mRNA synthesis and phosphatase treatment were not sufficient to prevent cell loss caused by the repeated transfection. Therefore, we further tested the use of the recombinant protein B18R—a soluble receptor of type I interferons—which has previously been used during the highly efficient synthetic mRNA-induced reprogramming of skin fibroblasts into induced pluripotent stem cells.[24]

Supplementation of culture media with B18R, significantly improved cell survival and attenuated the cell apoptosis induced by repeated transfection of synthetic mRNAs (**Supplementary Figure S1**). Therefore, in our further experiments, we used B18R supplementation with repeated daily transfection. This addition allowed us to achieve prolonged expressions of Pdx1, Neurogenin3, and MafA for at least 10 days (**Supplementary Figure S2a**). However, in order to limit the potential activation of innate immune response by the increased amount of synthetic mRNA exceeding 2 µg/ml, we used only 500 ng/ml of each mRNA (1,500 ng/ml of all three mRNAs) for repeated daily cotransfection, during the 10-day reprogramming period.

The coexpression of Pdx1, Neurogenin3, and MafA transcription factors was slightly increased following 10 days repeated daily cotransfection of all three synthetic mRNAs, in comparison with a single simultaneous transfection (**Supplementary Figure S2**). The rates of double-positive cells following 10 days repeated daily cotransfection were

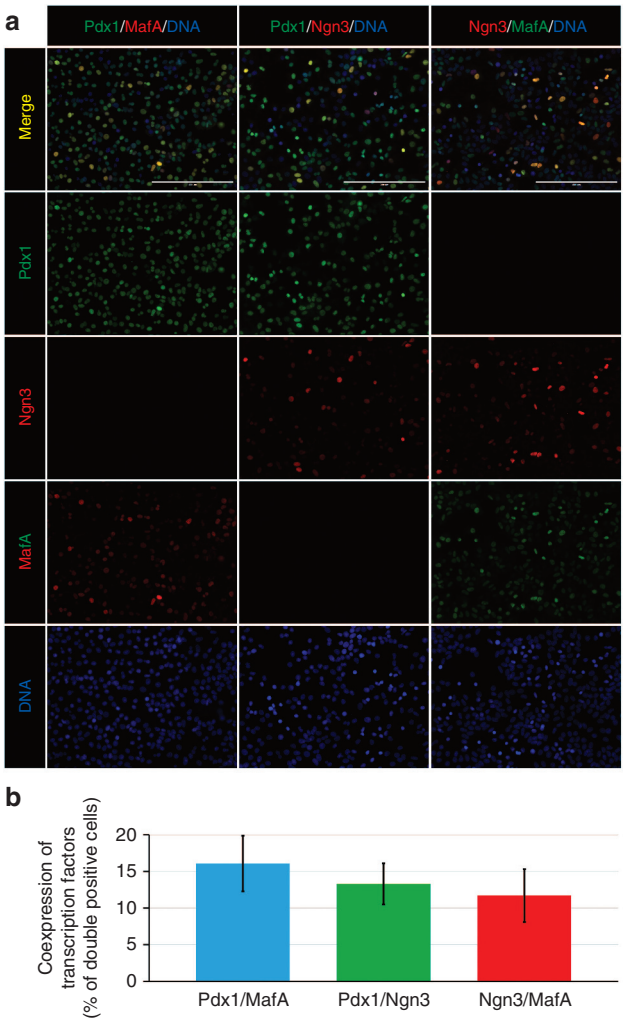

Figure 3 Transcription factors coexpression. (**a**, **b**) Immunofluorescence staining results showing coexpression of the transcription factors Pdx1, Neurogenin3 (Ngn3), and MafA following simultaneous transfection of AR42J cells with all three synthetic mRNAs at a dose of 500 ng of each mRNA/ml media. Double-positive cells are indicated by yellow color in the upper row. Cell nuclei (DNA) are stained blue with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride. Scale bars = 200 µm. Values are shown as mean ± standard deviation ($n = 4$).

20.5 ± 3.2% ($n = 4$) for Pdx1 and MafA, 17.8 ± 3.4% ($n = 4$) for Pdx1 and Neurogenin3, and 15.1 ± 5.1% ($n = 4$) for MafA and Neurogenin3 (**Supplementary Figure S2b**).

Reprogramming of pancreatic exocrine cells into insulin-producing cells using synthetic modified mRNAs encoding Pdx1, Neurogenin3, and MafA

We next investigated the potential of the synthetic modified mRNAs encoding Pdx1, Neurogenin3, and MafA for reprogramming pancreatic exocrine cells into insulin-producing cells. AR42J cells were transfected daily for 10 days with a combination of all three synthetic mRNAs, at doses of 500 ng/ml each, and cultured in serum-containing medium (denoted as a treatment group A) (**Figure 4a**). During the reprogramming period, cells began to express pancreatic hormones insulin and glucagon. However, the reprogramming efficiency was very low, with immunofluorescence staining showing only 3.5 ± 0.9% ($n = 4$) insulin-positive cells (**Figure 4b**). While the insulin expression was detected at the mRNA and protein levels, the expression of glucagon was detectable only at the mRNA level (**Figure 5**). The results of quantitative reverse transcription polymerase chain reaction further showed that repeated daily transfection with the synthetic mRNAs led to upregulation or induction of genes important for pancreatic β-cell differentiation (Pax4 and Nkx2.2) and function (Kir6.2, Sur1, Pcsk1, Pcsk2, and Glp1r) (**Figure 5**). However, some transcription factors (Isl1, Ngn3, Nkx6.1, and Pax6) and genes important for proper function (Glut2 and ZnT8) were upregulated only slightly or not at all (**Figure 5**). Detection of C-peptide by immunofluorescence staining (**Figure 6a**) revealed proper processing of prohormone peptide proinsulin into mature insulin and its byproduct C-peptide by the neuroendocrine endoproteases Pcsk1 and Pcsk2.

Serum exclusion from culture medium enhances reprogramming efficiency

We next attempted to improve the reprogramming efficiency by optimizing the culture conditions. It has previously been shown that exclusion of serum from culture medium can significantly improve reprogramming efficiency.[34] Indeed, our results showed that replacing fetal bovine serum with human serum albumin (denoted as a treatment group B) (**Figure 4a**) led to more efficient reprogramming, characterized by a greater proportion of insulin-positive cells (9.5 ± 1.7%, $n = 4$) and a higher insulin and C-peptide expression rates (**Figures 4b** and **6a**). These results were confirmed by quantitative reverse transcription polymerase chain reaction (**Figure 5**), which revealed further upregulation of genes important for a proper pancreatic β-cell function, including Glut2, Kir6.2, Pcsk1, and Pcsk2. However, the reprogrammed cells were not glucose-responsive as detected by inefficient glucose-stimulated insulin secretion (88 ± 12 versus 101 ± 15 pg insulin/µg DNA/ml) ($n = 5$) upon exposure to high glucose concentration (2.5 versus 20 mmol/l glucose) (**Figure 6b**).

Effect of DNA demethylation on cell reprogramming

Cell reprogramming efficiency depends on both the ectopic expression of reprogramming factors and the induction of endogenous genes. Thus, we further evaluated the effect of 5-Aza-2′-deoxycytidine on cell reprogramming and endogenous transcription factor expression. The chromatin-modifying agent 5-Aza-2′-deoxycytidine inhibits DNA methyltransferase activity, resulting in DNA demethylation, chromatin structure remodeling, and subsequently increased accessibility of genes for transcription factors—which is a necessary condition for gene expression activation.

Pretreatment of cells with 5-Aza-2′-deoxycytidine, followed by transfection with the synthetic mRNAs (denoted as a treatment group C) (**Figure 4a**) further improved reprogramming efficiency, as revealed by the increased proportion of insulin-producing cells (14.3 ± 1.9%, $n = 4$, **Figure 4b**); greater insulin gene expression; and upregulation of the functional genes Glut2 and Pcsk1, the transcription factors NeuroD and Pax6, and the maturation marker Urocortin3 (**Figure 5**).[35] Moreover, only the reprogramming protocol that included 5-Aza-2′-deoxycytidine pretreatment induced glucose-responsive reprogrammed cells,

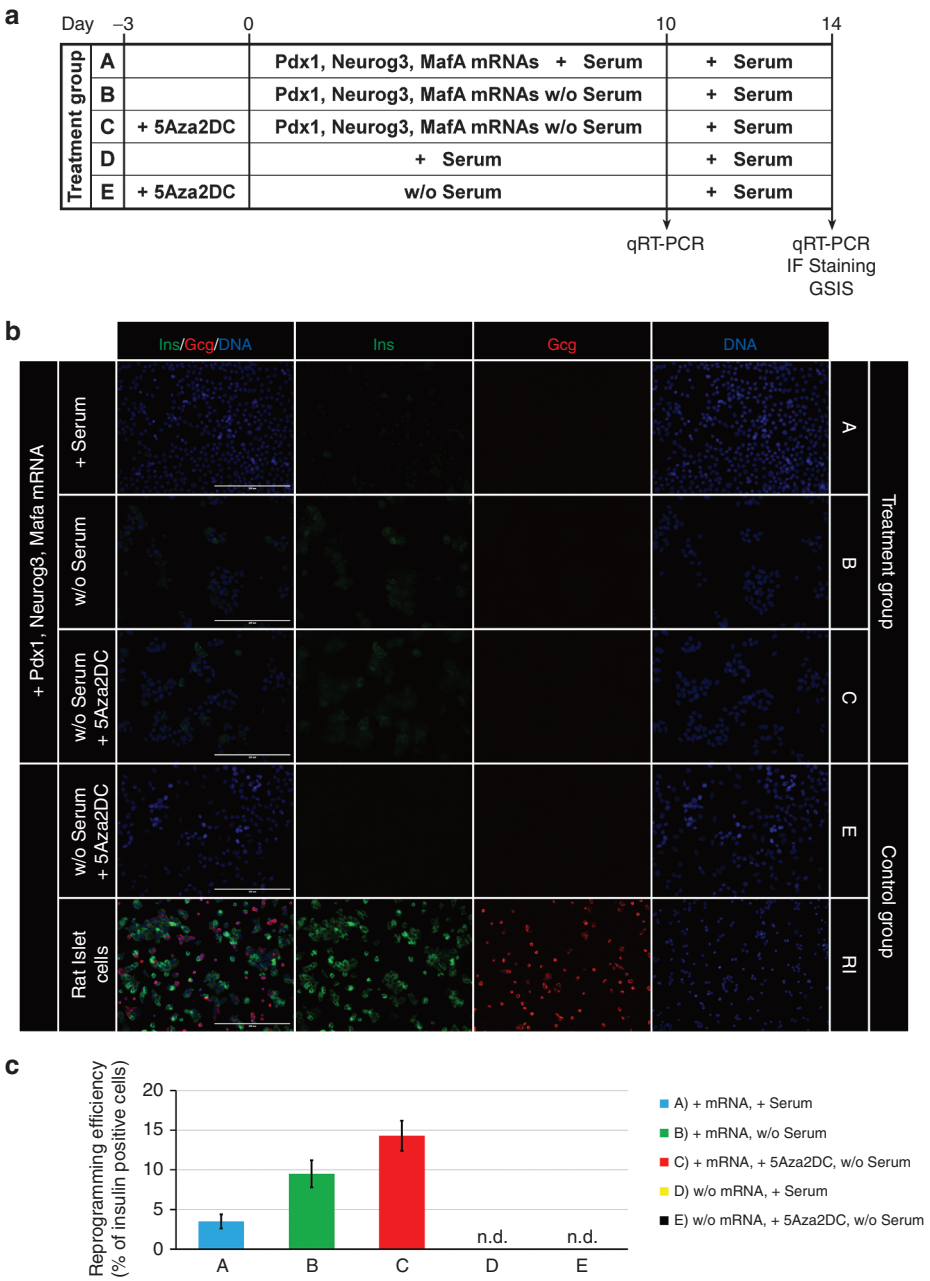

Figure 4 Scheme of the experimental design and evaluation of reprogramming efficiency. (**a**) Overview of the reprogramming protocol and subsequent analyses. Cell samples were divided into five groups based on culture conditions and the administration of all three reprogramming transcription factors (Pdx1, Neurogenin3, and MafA) for 10 days in the form of synthetic mRNAs at a dose of 500 ng of each mRNA/ml media. Cells were either cultured in serum-containing medium with mRNA transfection (treatment group A), cultured in serum-free medium with mRNA transfection (treatment group B), or pretreated for 3 days with 5-Aza-2′-deoxycytidine and cultured in serum-free medium with mRNA transfection (treatment group C). The expression profiles were compared with those of non-transfected AR42J cells that were either cultured in serum-containing medium (control group D), or pretreated for 3 days with 5-Aza-2′-deoxycytidine and cultured in serum-free medium (control group E) and of native rat pancreatic islets (control group RI). (**b**, **c**) Evaluation of reprogramming efficiency by immunofluorescence staining for the β-cell marker insulin (Ins) and the α-cell marker glucagon (Gcg). Insulin and glucagon expression was compared with non-transfected AR42J cells that were pretreated for 3 days with 5-Aza-2′-deoxycytidine and cultured in serum-free medium (control group E) and native rat pancreatic islet cells (control group RI). Cell nuclei (DNA) are stained blue with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride. Scale bars = 200 µm. Values are shown as mean ± standard deviation ($n = 4$). n.d., not detected.

and led to glucose-stimulated insulin secretion (842±72 versus 1,157±58 pg insulin/µg DNA/ml) ($n = 5$) upon exposure to high glucose concentration (2.5 vs. 20 mmol/l glucose) (Figure 6b). Insulin release under the basal glucose level (2.5 mmol/l glucose) was also induced by depolarizing agent potassium chloride (863±78 versus 1,025±66 pg insulin/µg DNA/ml) ($n = 5$), albeit at a lower extent than by high glucose concentration (Figure 6b).

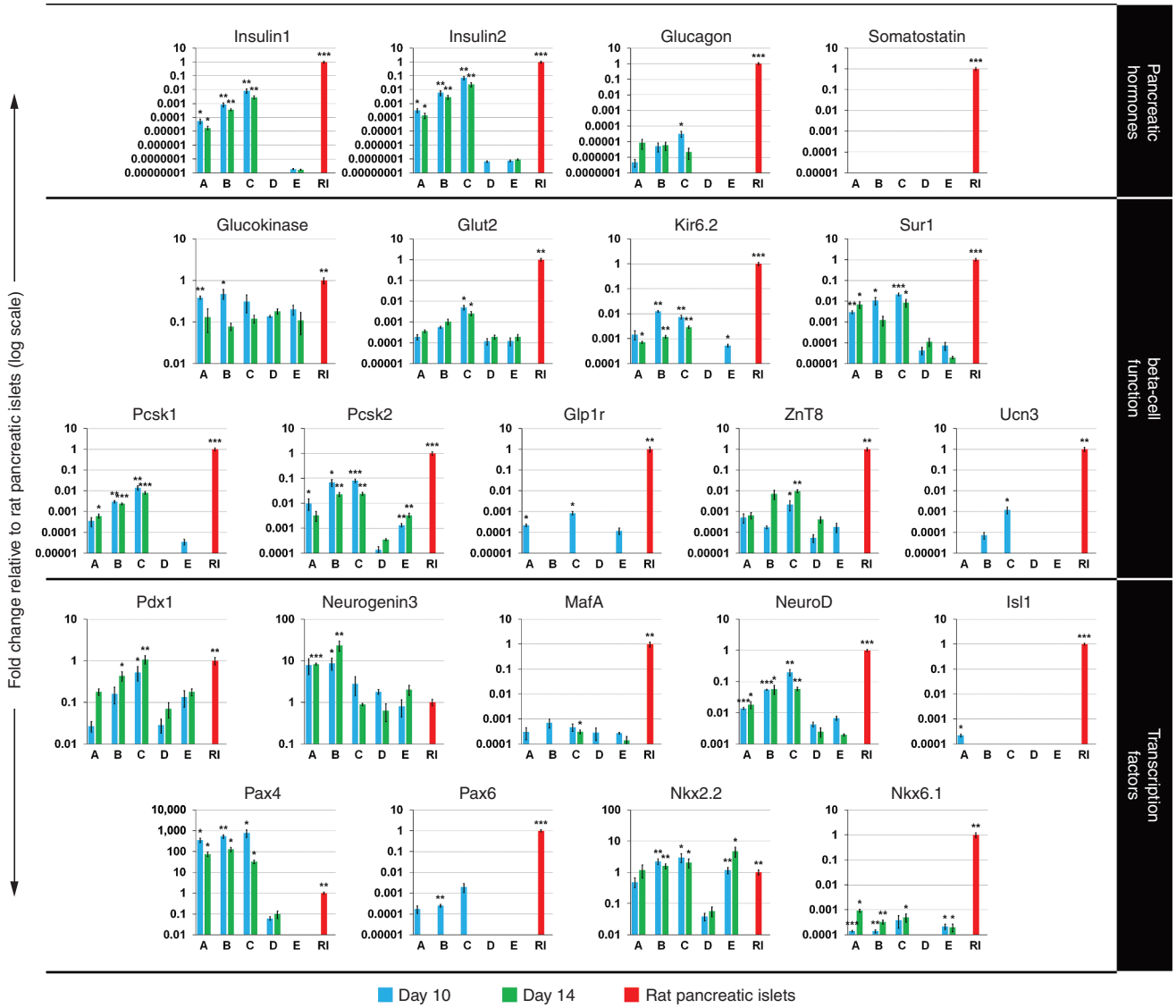

Figure 5 Gene expression profiles of reprogrammed cells were analyzed by quantitative reverse transcription polymerase chain reaction at the end of reprogramming (day 10—blue bars) and at 4 days after the last transfection with synthetic mRNAs (day 14—green bars). AR42J cells were treated with all three synthetic mRNAs (Pdx1, Neurogenin3, and MafA) for 10 days at a dose of 500 ng of each mRNA/ml media. Cells were either cultured in serum-containing medium with mRNA transfection (treatment group A), cultured in serum-free medium with mRNA transfection (treatment group B), or pretreated for 3 days with 5-Aza-2′-deoxycytidine and cultured in serum-free medium with mRNA transfection (treatment group C). The gene expression profiles were compared with those of native rat pancreatic islets (control group RI) and of nontransfected AR42J cells that were either cultured in serum-containing medium (control group D), or pretreated for 3 days with 5-Aza-2′-deoxycytidine and cultured in serum-free medium (control group E). Endogenous expressions of Pdx1, Neurogenin3, and MafA genes were determined using reverse primers specific for the 3′UTR (untranslated region) of each particular gene, which were not specific for synthetic mRNAs. The expression levels are presented relative to gene expression of rat pancreatic islets (normalized to 1). Values are shown as mean ± standard deviation ($n = 5$). Statistical analysis was performed using a two-tailed unpaired Student's *t*-test with Holm–Bonferroni correction. Samples were compared with nontransfected AR42J cells cultured in serum-containing medium (control group D). Asterisks indicate statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

However, in spite of improved reprogramming efficiency promoted by DNA demethylation, incomplete reprogramming of AR42J exocrine cells was also revealed by significantly lower insulin content (9.3 ± 1.3 ng insulin/μg DNA) (n = 5) in comparison with rat pancreatic islets (1,460.7 ± 268.1 ng insulin/μg DNA) (**Figure 6b**). Moreover, endogenous expression of Pdx1, Neurogenin3 and MafA transcription factors at protein level was not detected at the end of reprogramming period (day 14) (**Supplementary Figure S3**).

Discussion

Here, we report that pancreatic cells of exocrine origin can be transdifferentiated into insulin-producing cells using synthetic mRNAs encoding key transcription regulators of β-cell differentiation. To our knowledge, this is the first demonstration of

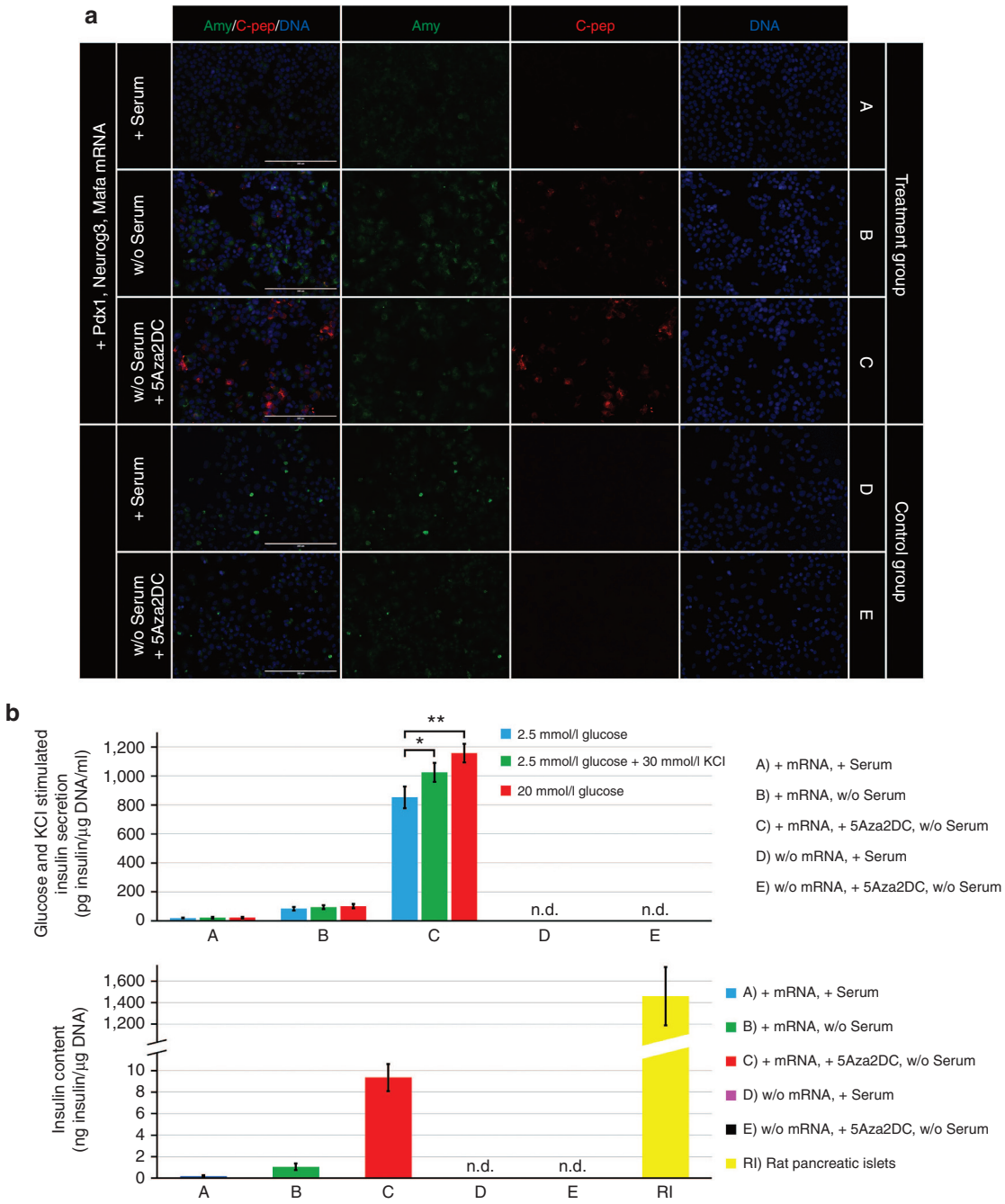

Figure 6 Reprogramming efficiency and determination of insulin secretion capacity and insulin content. (**a**) Reprogramming efficiency was evaluated by immunofluorescence staining for the exocrine marker amylase (Amy) and the β-cell marker C-peptide (C-pep). Cell nuclei (DNA) are stained blue with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride. Scale bars = 200 µm. (**b**) Glucose-stimulated insulin secretion of cell samples was determined by sequential 60-minute incubations at low (2.5 mmol/l) and high (20 mmol/l) glucose concentrations. The effect of depolarizing agent KCl on insulin secretion was determined by sequential 60-minute incubations at low (2.5 mmol/l) glucose concentration followed by low (2.5 mmol/l) glucose concentration with 30 mmol/l KCl. Insulin content in cell lysates was determined following KCl stimulated insulin secretion capacity test. Values are shown as mean ± standard deviation ($n = 5$). n.d., not detected. Statistical analysis was performed using a two-tailed unpaired Student's *t*-test. Asterisks indicate statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$.

direct reprogramming of pancreatic exocrine cells into insulin-producing cells using a nonintegrative approach involving intracellular delivery of synthetic mRNAs. Although the reprogrammed cells were not fully equivalent to primary β-cells, they shared important similarities. The reprogrammed cells produced mature insulin and its byproduct C-peptide by using the neuroendocrine endoproteases Pcsk1 and Pcsk2 that process the prohormone peptide proinsulin. Moreover, the reprogrammed cells expressed key elements of glucose-sensing mechanisms—including the glycolytic enzyme glucokinase, glucose transporter isoform-2 (Glut2), and the ATP-sensitive potassium channel subunits Sur1 and

Kir6.2—which are required to properly sense blood glucose level and for subsequent insulin secretion. Finally, the reprogrammed cells responded to glucose challenge with increased insulin secretion, although at a lower rate than the primary β-cells. However, the reprogrammed cells were not fully equivalent to primary β-cells, as shown by the low stimulatory index and the inability to increase insulin secretion upon membrane depolarization by KCl. Moreover, insulin content of reprogrammed cells was significantly lower in comparison with rat pancreatic islets. The immature phenotype of reprogrammed cells can be explained by insufficient expression of the transcription factors and of the genes responsible for the complex β-cell-specific expression program.

Cell transdifferentiation is characterized by suppression of the original expression program and induction of a newly acquired one,[36] which requires the expression of key regulatory transcription factors. Thus, reprogramming efficiency could potentially be improved by inducing the expressions of additional transcription factors. We propose that the transcription factors Nkx6.1, Pax6, and Isl1 are the most promising candidates for improving reprogramming efficiency, since their expressions were greatly limited or absent in our transdifferentiated cells. Each of these three transcription factors is active during the later phase of β-cell differentiation, and in mature β-cells.[37–39] Nkx6.1, Pax6, and Isl1 reportedly have positive effects on expressions of the insulin gene itself and of several key regulators of glucose-stimulated insulin secretion.[40–42]

Induction of the Nkx6.1 transcription factor may have been limited by possible repression by the exocrine cell-specific transcription factors Ptf1a and RbpJ.[43,44] Although, we only rarely observed amylase and C-peptide double-positive cells, we cannot exclude that the reprogrammed cells may have persistently expressed Ptf1a and RbpJ. On the other hand, Pax6 and Isl1, are downstream targets of the Neurogenin3 transcription factor.[39,45] Thus, it seems that the ectopic Neurogenin3 expression by reprogrammed cells was insufficient to induce endogenous expressions of Pax6 and Isl1. The limited Pax6 expression by our reprogrammed cells is in agreement with previous findings in insulin-secreting cells derived from human pancreatic ductal cells.[46] That study also revealed insufficient induction of endogenous Pax6 expression upon reprogramming induced by Pdx1, Neurogenin3, and MafA, and reported that ectopic Pax6 expression was required to enhance the expressions of insulin and other β-cell functional genes.

The epigenetic status of the transdifferentiated cells may have also influenced the induction of endogenous gene expression. Activation of gene expression during cellular differentiation requires remodeling of the gene-specific DNA chromatin structure from transcriptionally inactive heterochromatin into active euchromatin.[47] Thus, inappropriate chromatin remodeling can lead to insufficient induction of endogenous gene expression. Our results showed that the chromatin modifying agent 5-Aza-2′-deoxycytidine positively impacted cellular reprogramming and upregulation of gene expression. However, 5-Aza-2′-deoxycytidine only modulates DNA methylation status, not any other possible epigenetic modifications.

Gene expression can also be limited by repressive modifications of histone proteins that substantially impact chromatin structure. For example, the trimethylation of lysine 27 at histone H3 (H3K27) induces formation of an inactive heterochromatin structure.[47,48] A recent study comparing histone modifications between pancreatic exocrine cells and β-cells demonstrated one such H3K27 repressive modification of the Nkx6.1, Pax6, and Isl1 genes in pancreatic exocrine cells.[48] Furthermore, this same repressive H3K27 modification was detected in genes important for β-cell function following the *in vitro* differentiation of embryonic stem cells into insulin-producing cells.[49] The Glp1r and Urocortin3 genes were among those marked with a repressive modification, and were also inefficiently induced in our reprogrammed cells. The same previous work demonstrated the importance of proper chromatin modifications on gene expression, by showing the effect of an *in vivo* terminal differentiation in embryonic stem cell-derived cells. The *in vitro* terminally differentiated insulin-producing cells were associated with repressive histone modifications and with insufficient induction of genes important for β-cell function. On the other hand, *in vivo* terminal differentiation of embryonic stem cell-derived cells into insulin-producing cells induced permissive histone modifications and significantly higher expressions of the β-cell functional genes.[49]

Our reprogrammed insulin-producing cells did not resemble the so-called polyhormonal cells that produce insulin along with the other pancreatic hormones glucagon and somatostatin.[29] Therefore, we assume that our reprogramming protocol induced transdifferentiation directly to the β-like cell phenotype. During reprogramming, we observed substantial induction of the Pax4 transcription factor, which is transiently overexpressed during the early phase of pancreatic endocrine cells differentiation.[50] Pax4 restricts endocrine cell differentiation into the β- and δ-cell lineages via repression of the α-cell-specific transcription factor Arx.[51] Moreover, Pax4 and the transcription factor Nkx2.2 further specify the differentiation of endocrine progenitors into the β-cell phenotype.[52] While the endogenous expression of Pax4 transcription factor was induced following the reprogramming period, Nkx2.2 is naturally expressed by AR42J cells, and its expression was only slightly upregulated by the reprogramming factors. Further specification of AR42J cells into β-like cell phenotype could be promoted by the Pdx1 transcription factor that activates genes essential for β-cell identity and represses those associated with α-cell identity. Therefore, the ectopic overexpression of Pdx1 transcription factor, that is also naturally expressed by AR42J cells, can further stimulate the reprogramming of AR42J cells into β-like cell lineage. In addition to the transcription factors that are active during the early phase of β-cell differentiation, we also observed slight induction of the β-cell maturation marker Urocortin3 (ref. 35) at the end of the reprogramming period. Therefore, we assume that our reprogrammed insulin-producing cells resemble partially differentiated immature β-cells. This immature phenotype could be caused by insufficient induction of additional transcription factors such as MafA, that are responsible for the final maturation and proper function of pancreatic β-cells. Limited endogenous expression of MafA, which was significantly under-expressed in comparison with the native β-cells, can be explained by insufficient induction of Nkx6.1, Pax6, and Isl1 transcription factors that all positively regulate the MafA expression.[33,36–38]

Previous studies have reported the reprogramming of exocrine cells into insulin-producing cells using adenoviral vectors.[11–15,36] Although adenoviral vectors are highly efficient in the delivery and expression of introduced genes, the application of this process is limited by the potential for insertional mutagenesis,[18,19] and by the prolonged persistence in infected cells that does not allow modulation of the reprogramming process.[46] The presently described mRNA-based reprogramming resolves all of these issues. The mRNA chemical structure eliminates the risk of insertional mutagenesis or any other effect on cellular DNA. Moreover, the intracellular stability of mRNA is limited by permanent endogenous degradation, such that synthetic mRNA establishes only transient expression of the encoded gene. The use of synthetic mRNAs to induce temporal and sequential expression of different combinations of reprogramming factors allows to mimic the natural cellular differentiation process, in which some transcription factors are expressed only transiently while others are expressed over longer period of time.[39,44,45] Moreover, appropriate transcription factor stoichiometry can be achieved at different stages of cellular reprogramming.

On the other hand, disadvantages of mRNA-based reprogramming may include the need for repeated transfection and the potential cytotoxic effects of the synthetic mRNA. However, these issues could potentially be overcome by mRNA sequence optimization to improve the stability and translation efficiency, consequently reducing the required dose of mRNA.[53] Elimination of the cytotoxic effects of synthetic mRNA may also be promoted by high-performance liquid chromatography purification. These cytotoxic effects are mainly caused by aberrant byproducts formed during *in vitro* mRNA synthesis.[54,55] Highly efficient high-performance liquid chromatography purification can substantially reduce the amount of such byproducts in the final mRNA preparation, consequently eliminating cytotoxic effects and activation of the innate immune response by transfected cells.[55] It is worth noting that we did not test any of these possible improvements of synthetic mRNA in our present study. We only used the vaccinia virus B18R receptor of type I interferons to eliminate innate immune response activation. B18R application allowed us to achieve long-term repeated transfection of synthetic mRNA and to eliminate its cytotoxic effects. However, the addition of sequence optimization and high-performance liquid chromatography purification to our protocol would likely further improve the reprogramming efficiency and reduce the negative side effects.

Our present results demonstrate that using synthetic mRNAs encoding pancreatic transcription factors to reprogram pancreatic exocrine cells into insulin-producing cells, could represent a safe and promising approach for cell-based diabetes therapy. However, there remains a need for further optimization of the synthetic mRNAs, the culture conditions, and the combination of transcription factors to achieve efficient reprogramming into insulin-producing cells that are functionally equivalent to the native β-cells.

Materials and Methods

Construction of DNA Templates. **Figure 1a** shows the scheme for the production of DNA template constructs and subsequent RNA synthesis. All oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The **Supplementary Note S1** includes the oligonucleotide sequences used for DNA template construction. The Pdx1, Neurogenin3, and MafA coding regions were derived by reverse transcription of mRNA isolated from primary rat pancreatic islet cells, using gene-specific primers (**Supplementary Table S1**) and the AccuScript High-Fidelity 1st Strand cDNA Synthesis Kit (Agilent, Santa Clara, CA), following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of cDNA was performed using the same gene-specific primers and Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA), following the manufacturer's instructions. DNA template constructs were prepared using the pAcGFP1-N3 vector (Clontech, Mountain View, CA) along with the gBlock gene fragment (IDT) that contains sequences encoding the T7 RNA polymerase promoter site, the 5′ untranslated region (UTR) of the rat β-globin gene, two *Pst*I cloning sites, and the 3′UTR of the human β-globin gene. The gBlock gene fragment was inserted into the *Bam*HI and *Nhe*I (New England Biolabs) sites of the linearized pAcGFP1-N3 vector by homologous recombination, using the In-Fusion PCR cloning kit (Clontech), following the manufacturer's instructions. Then, the pAcGFP1-N3 vector with the integrated gBlock gene fragment was further linearized using the *Pst*I restriction enzyme (New England Biolabs). The In-Fusion PCR cloning kit was then used to insert cDNA of each transcription factor coding region into the *Pst*I-linearized vector. To verify the DNA sequence of the prepared vectors, we used the BigDye Terminator v3.1 Cycle Sequencing Kit with a 3130 Genetic Analyzer (Life Technologies, Grand Island, NY).

The *Nhe*I restriction enzyme (New England Biolabs) was used to excise a DNA template encoding the T7 RNA polymerase promoter site, the 5′UTR of the rat β-globin gene, the transcription factor open reading frame, and the 3′UTR of the human β-globin gene from the vector. This excised fragment was purified by agarose gel electrophoresis and the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). Isolated DNA fragment was PCR amplified with DNA template-specific primers (**Supplementary Table S2**) and Q5 High-Fidelity DNA Polymerase (New England Biolabs), following the manufacturer's instructions. The final PCR product was purified as described above and quantified by Qubit fluorometer (Life Technologies).

Synthesis of mRNA. RNA was synthesized using a T7 mScript Standard mRNA Production System (CELLSCRIPT, Madison, WI), with 20-µl reactions containing 2 µg of purified DNA template. We used a custom ribonucleotide blend comprising 3′-0-Me-m7G(5′)ppp(5′)G ARCA cap analog, pseudouridine triphosphate, 5-methylcytidine triphosphate (TriLink Biotechnologies, San Diego, CA), adenosine triphosphate, and guanosine triphosphate (New England Biolabs). The final reaction mixture contained 6 mmol/l ARCA cap analog, 3.0 mmol/l adenosine triphosphate, and 1.5 mmol/l of each the other nucleotides. Reactions were incubated for 1 hour at 37 °C and treated with DNase following the manufacturer's instructions. Next, RNA was purified via ammonium acetate precipitation, and treated with Antarctic phosphatase (New England Biolabs) for 2 hours at 37 °C to remove residual

5′-triphosphates. Treated RNA was again purified by ammonium acetate precipitation and polyadenylated for 2 hours at 37 °C using the Poly(A) Polymerase, Yeast (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Finally, the polyadenylated RNA was purified with the MEGAclear Transcription Clean-Up Kit (Life Technologies), diluted with RNAsecure Resuspension Solution (Life Technologies), and quantified by Qubit fluorometer. Synthetic mRNA quality was assessed using the Agilent RNA 6000 Nano Kit with an Agilent 2100 Bioanalyzer (Agilent).

Cell culture. The rat pancreatic exocrine cell line AR42J (Sigma-Aldrich) was cultured in Ham's F-12K medium (Life Technologies) containing 15% fetal bovine serum (Sigma-Aldrich) and 1% GlutaMAX supplement (Life Technologies). Cells were plated at 1×10^4 cells per well in 96-well-culture tissue dishes (Greiner Bio-One, Frickenhausen, Germany) on an extracellular matrix derived from the human bladder carcinoma cell line HTB-9, which was prepared as previously reported with a slight modification:

HTB-9 cells (American Type Culture Collection, Manassas, VA) were cultured in 96-well plates with Roswell Park Memorial Institute medium (Sigma-Aldrich) containing 10% fetal bovine serum and 1% GlutaMAX supplement. Cells were grown to confluence and cultured for an additional 3 days to allow extracellular matrix deposition. To decellularize the culture wells while leaving the intact extracellular matrix attached to the well surface, media was aspirated and each well was incubated for 5 minutes at 37 °C with 100 µl distilled water containing 20 mmol/l NH_4OH and 0.1% Triton X-100 (Sigma-Aldrich). The NH_4OH solution was then triturated four times and aspirated. Plates were inspected under microscope to ensure cell removal, and were washed five times with 37 °C phosphate-buffered saline (PBS) prior to seeding of AR42J cells.

During cell reprogramming, the AR42J cells were cultured in either serum-containing or serum-free Ham's F-12K medium (**Figure 4a**). Serum-free Ham's F-12K medium was supplemented with 0.5% human serum albumin, 1% insulin-transferrin-selenium, 1% Eagle's Minimum Essential Medium (MEM) nonessential amino acids (Life Technologies), 50 ng/ml epidermal growth factor, 10 ng/ml fibroblast growth factor 2, and 80 ng/ml insulin-like growth factor (PeproTech, Rocky Hill, NJ). Cell samples pretreated with 5-Aza-2′-deoxycytidine were cultured in serum-containing Ham's F-12K medium supplemented with 500 nmol/l 5-Aza-2′-deoxycytidine diluted in dimethyl sulfoxide (Sigma-Aldrich) for 3 days prior to reprogramming.

RNA transfection. RNA transfection was carried out using Lipofectamine MessengerMAX mRNA Transfection Reagent (Life Technologies). With Opti-MEM basal media (Life Technologies), synthetic mRNA was diluted to a concentration of 20 ng/µl and Lipofectamine MessengerMAX mRNA Transfection Reagent was diluted 33×. Diluted mRNA and transfection reagent were pooled 1:1 and incubated at room temperature for 5 minutes before being dispensed to the culture media. RNA transfections were performed in either serum-containing or serum-free Ham's F-12K medium, both supplemented with 200 ng/ml B18R interferon inhibitor (eBioscience, San Diego, CA).

Immunostaining. Cells were washed in Hank's Balanced Salt Solution (Sigma-Aldrich) and fixed in 4% paraformaldehyde for 15 minutes. Fixed cells were washed with PBS and blocked/permeabilized by a 30-minute incubation at room temperature with PBS containing 5% donkey serum (Sigma-Aldrich) and 0.3% Triton X-100 (Sigma-Aldrich). The cells were then stained in blocking buffer with primary antibodies for 30 minutes at 37 °C, washed, and then stained with secondary antibodies for 30 minutes at 37 °C with protection from light. Cell nuclei were stained for 15 minutes at room temperature with NucBlue Fixed Cell ReadyProbes Reagent (Life Technologies) diluted 1:10 in PBS. The following primary antibodies were used: rabbit anti-Pdx1 (1:200), rabbit anti-MafA (1:200), rabbit anti-insulin (1:300), mouse anti-C-peptide (1:100), mouse anti-glucagon (1:200) (Abcam, Cambridge, United Kingdom), mouse anti-Neurogenin3 (1:800), mouse anti-Pdx1 (1:400) (Developmental Studies Hybridoma Bank, Iowa City, IA), and rabbit anti-α-amylase (1:200) (Sigma-Aldrich). The secondary antibodies were donkey anti-mouse or donkey anti-rabbit IgG Alexa Fluor 555 and/or Alexa Fluor 647 (Life Technologies) at a 1:400 dilution. Images were acquired with the EVOS FL Auto Cell Imaging System (Life Technologies). Positive cells was quantified from at least ten visual fields (with 100× magnification) using the EVOS FL automatic cell counting tool.

Gene expression analysis. Total RNA was isolated using the RNeasy Mini Plus kit (Qiagen, Valencia, CA). The RNA was then treated for 1 hour at 37 °C with Turbo DNase (Life Technologies), repurified using RNA Clean & Concentrator-5 (Zymo Research, Irvine, CA), and quantitated using a Qubit fluorometer. Next, 500 ng of isolated RNA was reverse-transcribed at 50 °C for 60 minutes with random hexamer and anchored oligo dT primers (5:1 ratio) using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Rotkreuz, Switzerland) following the manufacturer's instructions. The generated cDNAs were analyzed by PCR using FastStart Universal SYBR Green Master Rox (Roche) with gene-specific primers (Integrated DNA Technologies) for each detected mRNA (**Supplementary Table S3**). PCR started with 10 minutes at 95 °C, which was followed by 40 cycles of 15 seconds at 95 °C (denaturation) and 1 minute at 62 °C (annealing/extension). Reactions and data analysis were carried out using a ViiA 7 Real-Time PCR System (Life Technologies). All samples were assayed in triplicates. Fold-changes in gene expression were determined using the ΔΔCT method, with normalization to β-actin expression.

Apoptosis assay. To test the synthetic mRNA for cytotoxic effects we used CellEvent Caspase-3/7 Green ReadyProbes Reagent (Life Technologies), following the manufacturer's instructions. AR42J cells were cultured in a 96-well plate, and transfected twice for 2 days with a mixture of Pdx1, Ngn3, and MafA synthetic mRNAs (1:1:1 ratio) at a total dose of 1–2 µg/ml. On the third day, we analyzed induction of apoptosis by the synthetic mRNA. CellEvent Caspase-3/7 Green Reagent and NucBlue Live ReadyProbes Reagent (Life Technologies) were added to each well and incubated at 37 °C for 30 minutes in a CO_2 incubator. Next, the cell samples were washed three times with PBS, and images were acquired using an

EVOS FL Auto Cell Imaging System (Life Technologies). The number of apoptotic cells and total cell number were determined from at least 10 visual fields (at 100× magnification) using the EVOS FL automatic cell counting tool.

Glucose-stimulated insulin secretion assay. Cell samples were cultured in 24-well plates and then washed three times with 0.5 ml Krebs solution (128 mmol/l NaCl, 5 mmol/l KCl, 2.7 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 1 mmol/l Na_2HPO_4, 1.2 mmol/l KH_2PO_4, 5 mmol/l $NaHCO_3$, and 10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) containing 0.1% human serum albumin and 2.5 mmol/l glucose (low-glucose solution). To normalize insulin secretion, the cell samples were then preincubated for 1 hour in the low-glucose solution. Then the low-glucose solution was refreshed and the cell samples were again incubated for 1 hour. A 250-µl sample of the low-glucose solution supernatant was aspired, centrifuged at 10,000 g for 5 minutes at 4 °C, and then immediately frozen and stored at –80 °C until analysis. The cell samples were washed three times with 0.5 ml of Krebs-Ringer solution containing 20 mmol/l glucose (high-glucose solution) or 2.5 mmol/l glucose and 30 mmol/l KCl (high KCl solution), and incubated for an additional hour. A 250-µl sample of the high-glucose solution or high KCl solution supernatant was aspired, centrifuged at 10,000 g for 5 minutes at 4 °C, and immediately frozen and stored at –80 °C until analysis.

For analysis, the cells were lysed in 0.3 ml RIPA buffer (Sigma-Aldrich) and DNA content was determined using a Qubit fluorometer. In samples from the glucose-stimulated insulin secretion assay and cell lysates, insulin content was determined using the Insulin 125I RIA kit (MP Biomedicals, Orangeburg, NY) according to the manufacturer's instructions. All incubation steps were performed at 37 °C in a CO_2 incubator, and all solutions were equilibrated to 37 °C prior to use.

Statistical analysis. Statistical analyses were performed using a two-tailed unpaired Student's *t*-test with Holm–Bonferroni correction using GraphPad software. *P* values of <0.05 were considered to indicate statistically significant differences. The numbers of independent experiments performed are indicated in the text. Mean values are presented with standard deviations in the format (mean ± standard deviation).

Supplementary material

Figure S1. Evaluation of the effect of recombinant B18R interferon inhibitor on prevention of apoptosis upon repeated transfection of exocrine cells with synthetic mRNAs.
Figure S2. Transcription factors co-expression after repeated daily transfections.
Figure S3. Endogenous transcription factors co-expression after repeated daily transfections.
Table S1. Primers used for reverse-transcription and PCR amplification of transcription factors cDNAs and PCR amplification of DNA templates used for *in vitro* transcription.
Table S2. Primers used for PCR amplification of DNA templates for *in vitro* transcription.
Table S3. Primers used for qRT-PCR gene expression analysis.
Note S1. Oligonucleotide sequences of DNA constructs and templates used for *in vitro* transcription.

Supplementary Information

Acknowledgments T.K. managed the project, designed the experiments, executed the experiments, analyzed and interpreted the data, and wrote the paper. I.L. and S.L. executed the experiments and analyzed the data. L.K. executed the experiments. F.S. interpreted the data and wrote the paper. This work was supported by an IGA grant (project reference number NT12190-5/2011) from the Ministry of Health, Czech Republic. We thank Milan Jirsa for discussions and critical reading of this manuscript and Jan Kriz for providing samples of rat pancreatic islets. The authors declare no conflict of interest.

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Original Article

Activation of the Jak/Stat Signalling Pathway by Leukaemia Inhibitory Factor Stimulates Trans-differentiation of Human Non-Endocrine Pancreatic Cells into Insulin-Producing Cells

(diabetes mellitus / insulin / pancreas / beta cell / islets / stem cells / leukaemia inhibitory factor / differentiation / Notch / Jak/Stat)

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Abstract. Differentiation of pancreatic β-cells is regulated by a wide range of signalling pathways. The aim of our current work was to evaluate the effect of the Jak/Stat signalling pathway on the differentiation of human non-endocrine pancreatic cells into insulin-producing cells. Activation of the Jak/Stat signalling pathway by leukaemia inhibitory factor (LIF) stimulated differentiation of C-peptide-negative human non-endocrine pancreatic cells into insulin-producing cells in 6.3 ± 2.0 % cells (N = 5) and induced expression of pro-endocrine transcription factor neurogenin 3, Notch signalling pathway suppressor HES6 and stimulator of β-cell neogenesis REG3A. The expression of the *REG3A* gene and increased rate of differentiation into insulin-producing cells (10.2 ± 2.1 %) were further stimulated by a combination of LIF with nicotinamide and dexamethasone. Glucose-stimulated (5 vs. 20 mM) C-peptide secretion confirmed proper insulin secretory function of trans-differentiated insulin-producing cells (0.51 vs. 2.03 pmol C-peptide/μg DNA, P < 0.05). Our results indicate that Jak/Stat signalling critically contributes to trans-differentiation of non-endocrine pancreatic cells into functional insulin-producing cells. The positive effect of the Jak/Stat signalling pathway on trans-differentiation is mediated by the key genes that activate differentiation of pancreatic β-cells.

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Abbreviations: bFGF – basic fibroblast growth factor, DAPI – 4,6-diamidino-2-phenylindole, EGF – epidermal growth factor, HES – hairy and enhancer of split, HNF6 – hepatocyte nuclear factor 6, IGF – insulin-like growth factor, ILCC – islet-like cell cluster, INGAP – islet neogenesis-associated protein, ITS – insulin transferrin selenium, LIF – leukaemia inhibitory factor, PBS – phosphate-buffered saline, REG3A – regenerating islet-derived 3 α protein, RT-PCR – reverse transcriptase polymerase chain reaction.

Introduction

Cell-based therapy of diabetes mellitus is an attractive approach to efficient treatment of all diabetic patients. In spite of the advances achieved in the field of regenerative medicine, a plentiful source of insulin-producing tissue is still unavailable. Adult pancreatic non-endocrine cells represent one of the potential alternative sources of newly formed insulin-producing cells applicable to the cell-based treatment of diabetes. Potentially, the non-endocrine pancreatic cells sharing a similar embryological origin and being easily available could be differentiated into β-like cells either *in vitro* or *in vivo*. However, the differentiation of pancreatic β-cells is regulated by a complex interplay of a wide range of growth factors, transcription factors and signalling pathways which is not fully understood so far. Transforming growth factor β (TGF-β), Notch and Hedgehog signalling pathways have been shown to play key roles in the development of pancreatic tissue and β-cell differentiation (Apelqvist et al., 1999; Hebrok et al., 2000; Goto et al., 2007). They not only transduce the external signals activated by different growth factors, but also regulate the expression and activity of key transcription factors of β-cell differentiation (Kim and Hebrok, 2001).

Another signalling pathway that has recently been identified as having an important role in β-cell differentiation is the Jak/Stat signalling pathway (Baeyens and Bouwens, 2008). Its activation induces expression of islet neogenesis-associated protein (INGAP) (Taylor-Fishwick et al., 2006). INGAP has been shown to stimulate generation of new islet cells *in vitro* as well as in adult animal models (Rosenberg et al. 2004). In other

experiments, the Jak/Stat signalling pathway had a positive effect on β-cell differentiation (Baeyens et al., 2005). Stimulation of the Jak/Stat pathway by a combination of leukaemia inhibitory factor (LIF) and epidermal growth factor (EGF) led to transient expression of neurogenin 3, a key transcription factor required for pancreatic endocrine cell differentiation (Baeyens et al., 2006).

During the isolation of human pancreatic islets only approximately 2 % of the pancreatic tissue representing the islets of Langerhans is finally used for clinical transplantation. Currently the remaining pancreatic tissue is discarded. Based on these encouraging reports in animal research, we decided to evaluate the effect of the Jak/Stat signalling pathway stimulation on β-cell differentiation using the human non-endocrine pancreatic tissue. Should the experimental results be confirmed, exocrine pancreatic tissue could represent a promising and available cell source for diabetes treatment.

Material and Methods

Tissue preparation

Human non-endocrine pancreatic cells were obtained from the remaining pancreatic tissue after islet isolation. The programme of isolation and transplantation of human pancreatic islets was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Teaching Hospital. Human islets and pancreatic tissue were isolated according to the previously described method (Linetsky et al., 1997). Briefly, islets were isolated from pancreata obtained from cadaveric donors (N = 5; mean age 43 ± 16 years). The pancreatic duct was perfused with a cold enzyme mixture containing Collagenase NB 1 Premium Grade and Neutral Protease NB (Serva, Heidelberg, Germany). Perfused pancreatic tissue was transferred to a modified Ricordi chamber and dissociated by gentle mechanical agitation and enzymatic digestion at 37 °C. Islets were purified with the use of continuous gradients of Biocoll (Biochrom, Berlin, Germany) in an aphaeresis system Cobe model 2991 (Gambro Czech Republic, Přerov, Czech Republic). The densities of the continuous gradient ranged from 1.065 to 1.090 g/ml. During centrifugation, islets migrated to the interface between 1.070–1.080 g/ml. The remaining cellular material from the islet-depleted fractions was pooled and further digested in Accutase solution (Sigma-Aldrich, Steinheim, Germany) for 20 min at 37 °C. Single-cell suspension was obtained after filtration through an 11-μm cell strainer and purification with the use of Biocoll continuous gradient in an aphaeresis system Cobe model 2991. The densities of the continuous gradient ranged from 1.030 to 1.100 g/ml. Cell suspension purified from the 1.050–1.080 g/ml interface was pooled, washed in Hank's solution (Sigma-Aldrich) and further processed.

Cell culture studies

Pancreatic cells isolated from islet-depleted pancreatic tissue were cultured for the first three days (stage 1) in DMEM medium containing 10% (v/v) KnockOut Serum Replacement, 1% (v/v) Insulin-Transferrin-Selenium A Supplement (ITS), 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine, 1% (v/v) nonessential amino acids, 0.1 mM 2-mercaptoethanol (all from Invitrogen, Paisley, UK), 10 ng/ml bFGF, 20 ng/ml EGF (both from Peprotech, Rocky Hill, NJ) and conditioned medium derived from neonatal fibroblast cell line Hs68 (LGC Promochem, Teddington, UK). Samples were divided into three groups based on the addition of LIF, nicotinamide and dexamethasone. Group 1 was supplemented only with human recombinant LIF (40 ng/ml) (Peprotech), group 2 was supplemented with human recombinant LIF (40 ng/ml) (Peprotech), nicotinamide 10 mM (Sigma-Aldrich) and dexamethasone (100 nM) (Sigma-Aldrich), group 3 served as a control group without any of the supplements. Culture medium was replenished daily during the first three days. Within the first three days the cells formed a cellular cluster further referred to as islet-like cell cluster (ILCC).

Afterwards, cells were cultured for another three days (stage 2) in CMRL medium containing 1% FCS, 10 μM SP600125, 10 μM SB 216763, 10 μM forskolin, 5 μg/ml fibronectin, 10 mM nicotinamide, 40 ng/ml Exendin-4 (all from Sigma-Aldrich) and 100 ng/ml IGF (Peprotech). Culture medium was replenished on the 2nd day of stage 2 culture period.

Reverse transcriptase polymerase chain reaction

Total RNA (from approximately 10^6 cells) was isolated by Rneasy Plus Mini Kit (Qiagen, Hilden, Germany) and treated with DNAse using RNase-Free DNase Set (Qiagen). Isolated RNA (1 μg) was reverse transcribed with Omniscript RT Kit (Qiagen) according to the manufacturer instructions. cDNA was amplified using HotStarTaq Master Mix Kit (Qiagen) and gene-specific primers. Annealing temperatures, number of cycles and product sizes are shown in Table 1. Total RNA from the islet fraction was used as positive control. PCR products were separated and visualized on 1% agarose gel containing ethidium bromide.

Immunocytochemistry

ILCCs collected at the end of the experiment were washed with PBS, fixed for 60 min in Bouin's solution (Sigma-Aldrich), rinsed with PBS, suspended in 2% agarose-PBS solution and centrifuged at 100 *g* for 3 min to form compact pellets. After overnight submersion in 30% sucrose (Sigma-Aldrich), ILCCs were embedded in OCT mounting medium TissueTek (Bayer Corp, Pittsburgh, PA), frozen in liquid nitrogen, and stored at -80 °C.

Table 1. Sequences of gene-specific primers and product size of cDNA products

Gene	Forward primer	Reverse primer	Number of cycles	Product size (bp)
Insulin	ccatcaagcacatcactgtcc	ccatctctctcggtgcagg	28	414
Glucagon	gcgagatttcccagaagagg	agcaggtgatgttgtgaagatg	28	198
PDX1	gcaccttcaccaccacctc	cttgttctcctccggctcc	30	202
Neurogenin 3	tctattcttttgcgccggtag	agtgccaactcgctcttagg	32	256
MAFA	ccaaagagcgggacctgta	cctggtgtccacgtcctgta	30	253
REG3A	cctggtgaagagcattggtaac	ttgggggaattaagcgaata	28	364
HES1	ctaccccagccagtgtcaac	atgtccgccttctccagc	30	193
HES6	tgaggatgaggacggctg	cgagcagatggttcaggag	30	350
HNF6	cgcaggtcagcaatggaag	gatgagttgcctgaattggag	30	535
GAPDH	ggagtcaacggatttggtcg	catgggtggaatcatattggaac	23	142

After several washes in PBS, 8 μm sections of frozen ILCC slides were incubated in blocking solution containing 10% (v/v) normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA) in 0.2% (v/v) Triton X-100, 0.1 M glycine (Sigma-Aldrich) and PBS for 1 h at room temperature to prevent unspecific binding. Incubation with primary antibodies in appropriate dilution was performed in a blocking solution for 1 h at 37 °C. The following primary antibodies were used at the 1 : 100 dilution: mouse anti-cytokeratin 19, mouse anti-C-peptide (both from Exbio, Vestec, Czech Republic) and rabbit anti-C-peptide (Cell Signaling, Danvers, MA). After intensive washing with PBS, sections were incubated with the specific secondary antibody diluted in the blocking solution for 1 h at 37 °C. The secondary antibodies were Alexa Fluor 555 donkey anti-mouse IgG and Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen). 4,6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at a concentration of 5 μg/ml was used to label the nuclei for 10 min at 37 °C. After rinsing with PBS, sections were mounted with antifade solution and examined with fluorescent microscope Olympus BX41 (Olympus, Tokyo, Japan).

C-peptide cell content and glucose-stimulated secretion

C-peptide release was measured by incubating 100 ILCCs in 1 ml of Krebs-Ringer solution containing 5 mM glucose for 1 h and then in 20 mM glucose solution for another 1 h. Cells were lysed in RIPA buffer (Sigma-Aldrich) and human C-peptide was determined using a C-peptide IRMA kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using Student's *t*-test. All data are presented as means ± SD. P values < 0.05 were considered significant. Evaluated null hypothesis was that LIF does not have a positive effect on differentiation of non-endocrine pancreatic cells into insulin-producing cells.

Results

In order to evaluate the possible contamination of non-endocrine pancreatic cell samples by insulin-positive β-cells we determined the ratio of insulin-positive β-cells in islet-depleted cell suspension. Samples of non-endocrine pancreatic cells contained 0.24 ± 0.07 % of pancreatic β-cells based on the dithizone (diphenylthiocarbozone) staining (Fig. 1) and immunofluorescence staining of C-peptide-positive cells (data not shown). Slight contamination of samples by β-cells was also confirmed by RT-PCR (Fig. 2). Although samples contained some β-cells, these cells did not proliferate during the stage 1 culture period. The number of β-cells even decreased within the first three days under all tested conditions to the ratio of 0.19 ± 0.06 % in the LIF--treated sample, 0.21 ± 0.04 % in the LIF-, nicotinamide- and dexamethasone-treated sample and 0.22 ± 0.06 % in the control sample.

Most of the cells from the initial cell suspension aggregated into ILCCs resembling islets of Langerhans within three days of the culture period in serum-free neonatal fibroblast-conditioned medium. Under all tested conditions ILCCs were formed mainly by cytokeratin-19-positive cells with the exception of the LIF-, nicotinamide- and dexamethasone-treated sample. The number of cytokeratin-19-positive cells was significantly lower in the LIF-, nicotinamide- and dexamethasone-treated sample (42.8 ± 3.7) in comparison with the LIF-treated (61.3 ± 5.2) and control (64.1 ± 4.9) samples (data not shown).

The expression of transcription factors that are involved in pancreatic endocrine cell differentiation (*PDX1*, neurogenin 3, *HNF6* and *MAFA* genes) and Notch signalling pathway (*HES1* and *HES6* genes) was also different between LIF-treated and control samples after three days of cultivation. In comparison with control samples, the expression of *PDX1*, *HNF6* and neurogenin 3 genes was significantly higher in the samples treated either with LIF alone or with LIF in combination with nicotinamide and dexamethasone. The level of the *HES6* gene expression was also significantly higher in the case of cultures treated with LIF alone or LIF-, nicotinamide- and dexamethasone-treated samples than in the control samples, while expression of the *HES1* gene

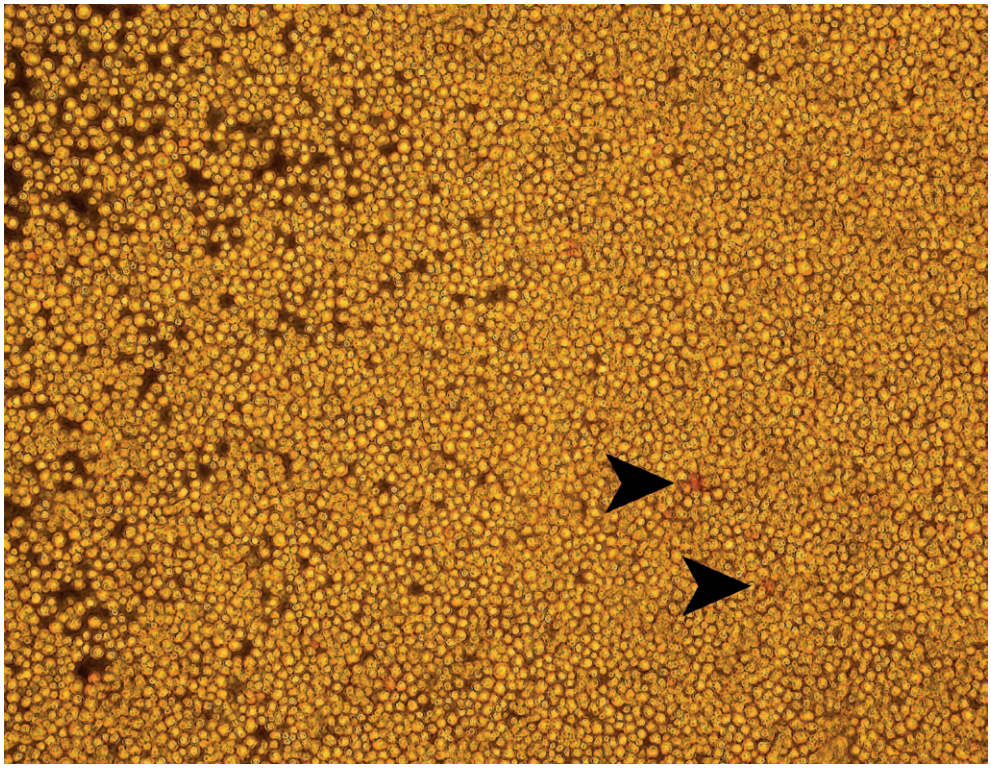

Fig. 1. Dithizone staining of cell suspension derived from dissociated islet-depleted pancreatic tissue. Red dithizone-stained insulin-positive cells (arrows).

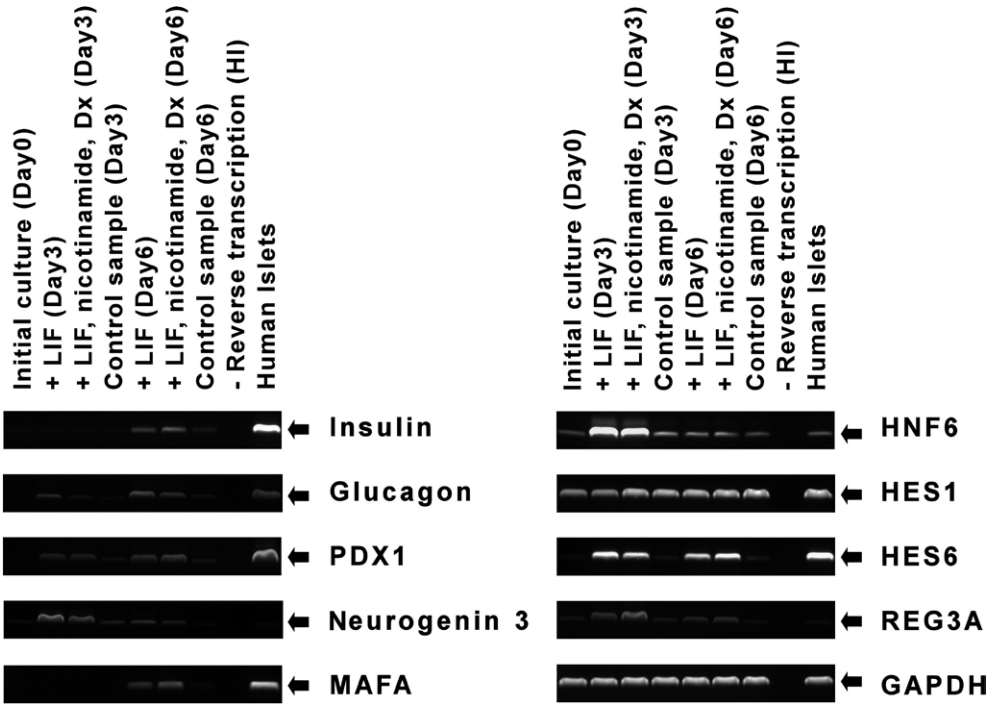

Fig. 2. Reverse transcriptase polymerase chain reaction analysis of gene expression during differentiation of pancreatic non-endocrine cells. RNA isolated from human pancreatic islets was used as a positive control. RNA isolated from human pancreatic islets without reverse transcription was used as a negative control.

was similar between all samples. The expression of the *REG3A* gene, a human analogue of hamster INGAP protein, was also significantly higher in the samples treated with LIF than in the control samples. The highest rate of *REG3A* expression was detected in the samples treated with a combination of LIF, nicotinamide and dexamethasone.

While the expression of insulin gene was almost undetectable, a minimal rate of the glucagon gene expression was detected in all samples with the highest rate in

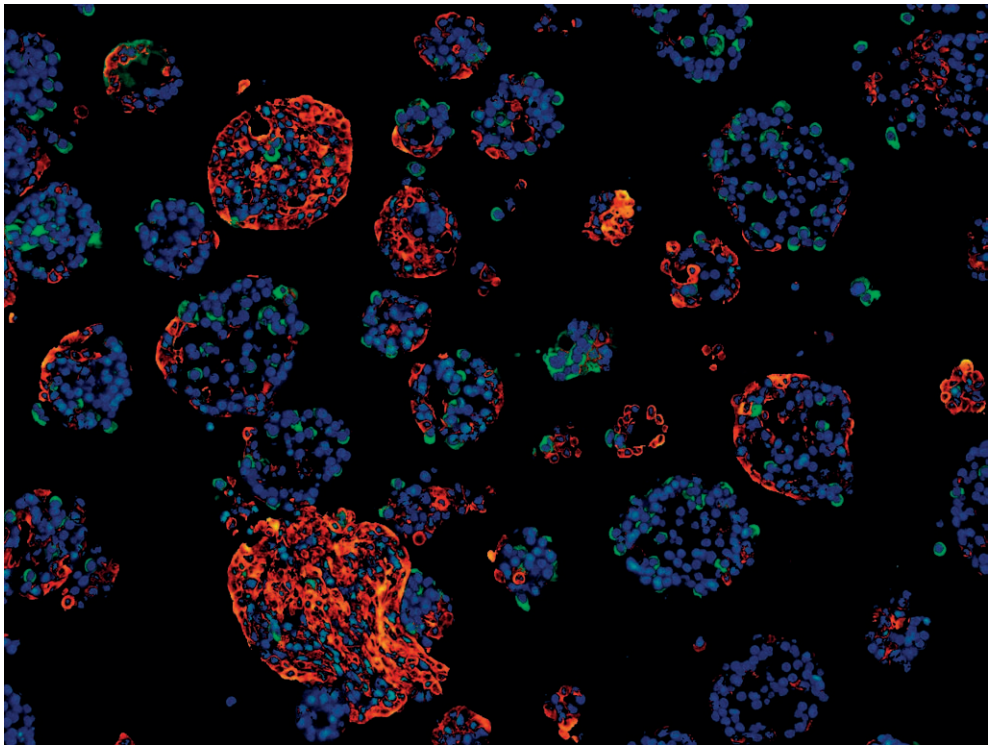

Fig. 3A. Immunofluorescence staining of cytokeratin-19 (orange) and C-peptide (green) in ILCCs treated with a combination of LIF, nicotinamide and dexamethasone during the first three days of the culture period. DAPI (blue) stain was performed as counter stain (magnification 100×).

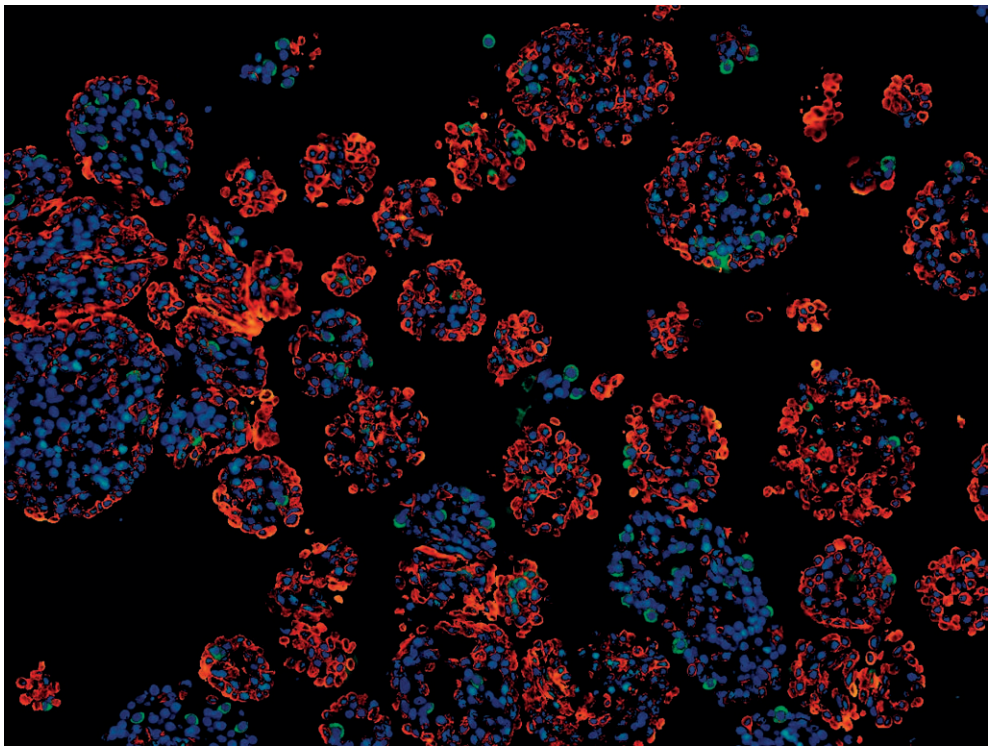

Fig. 3B. Immunofluorescence staining of cytokeratin-19 (orange) and C-peptide (green) in ILCCs treated with LIF during the first three days of the culture period. DAPI (blue) stain was performed as counter stain (magnification 100×).

the LIF-treated samples after three days of cultivation (Fig. 2).

Within the next three days during stage 2 of the differentiation protocol the cells differentiated into insulin-producing cells. The highest number of C-peptide-positive cells was detected in a sample previously treated with LIF, nicotinamide and dexamethasone (10.2 ± 2.1) (Fig. 3A), while LIF-treated (6.3 ± 2.0) (Fig. 3B) and

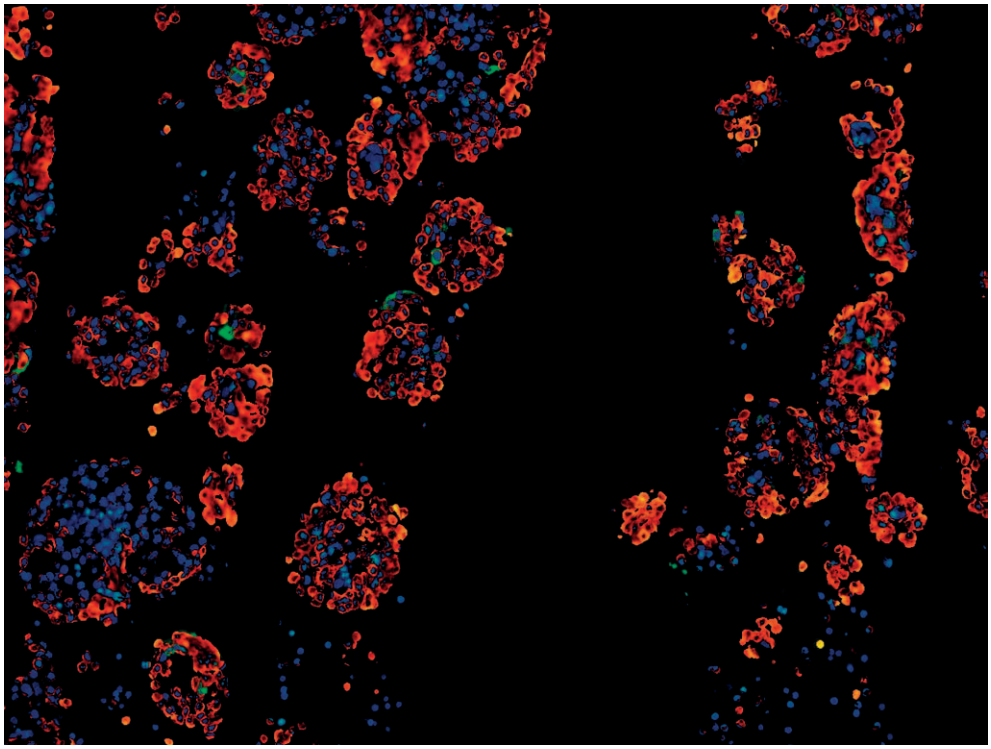

Fig. 3C. Immunofluorescence staining of cytokeratin-19 (orange) and C-peptide (green) in ILCC control sample untreated with any of the tested compounds during the first three days of the culture period. DAPI (blue) stain was performed as counter stain (magnification 100×).

control samples (3.5 ± 1.3) (Fig. 3C) had a lower number of C-peptide-positive cells based on the immunofluorescence staining. The higher rate of β-cell differentiation in samples treated with a combination of LIF, nicotinamide and dexamethasone was confirmed by RT-PCR (Fig. 2). The expression of the *PDX1* transcription factor gene in samples treated with LIF was still significantly higher in comparison with control samples, while the expression of transcription factor neurogenin 3, *HES6* and *REG3A* genes that were induced in LIF-treated samples declined during stage 2. The expression of *REG3A* also declined after withdrawal of either LIF alone or a combination of LIF, nicotinamide and dexamethasone during the last three days of the culture period.

Differentiation of islet-depleted non-endocrine pancreatic cells into insulin-producing β-cells was additionally confirmed by analysis of the C-peptide cell content and glucose-stimulated secretion (Fig. 4). The highest C-peptide content was detected in samples treated with LIF, nicotinamide and dexamethasone (23.4 ± 4.6 pmol C-peptide/μg DNA) followed by the LIF-treated sample (13.8 ± 3.2 pmol C-peptide/μg DNA). In the control sample, the C-peptide content was significantly lower (6.2 ± 2.3 pmol C-peptide/μg DNA) (Fig. 4). The insulin secretory capacity of differentiated ILCC cells was confirmed by the glucose-stimulated C-peptide secretion test. In response to glucose stimulation (5 vs. 20 mM) ILCCs treated with a combination of LIF, nicotinamide and dexamethasone secreted 0.51 vs. 2.03 pmol C-peptide/μg DNA.

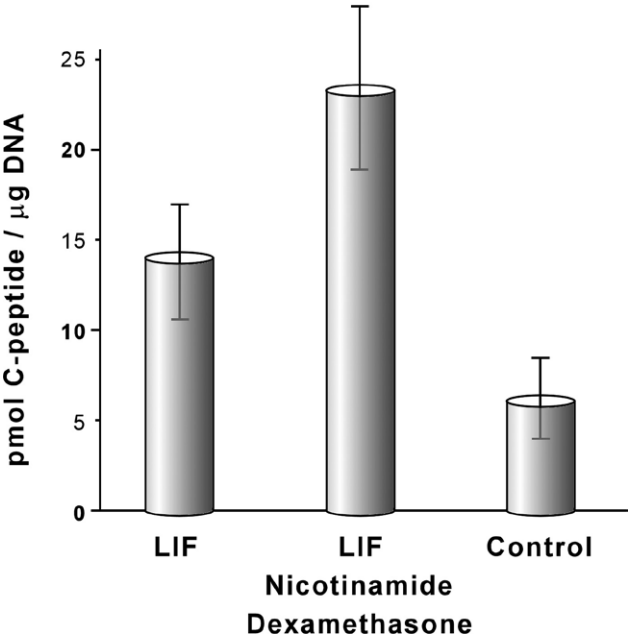

Fig. 4. C-peptide content of non-endocrine pancreatic cells treated with LIF or with a combination of LIF, nicotinamide and dexamethasone and control sample as determined by IRMA.

Discussion

In our current report we have shown that LIF stimulates differentiation of human non-endocrine pancreatic cells into insulin-producing cells. The positive effect of

LIF treatment on β-cell differentiation was further enhanced by co-treatment with nicotinamide and dexamethasone. Although we were not able to determine the underlying mechanism of LIF-stimulated β-cell differentiation, we assume that such a positive effect is at least partially mediated by the induction of the regenerating islet-derived 3 α protein (REG3A) expression. *REG3A* and its hamster analogue *INGAP* belong to a superfamily of *Reg* genes, which are associated with β-cell proliferation and regeneration (Fleming and Rosenberg, 2007). While members of the Reg1 family stimulate β-cell proliferation (Watanabe et al., 1994), members of the Reg3 family are associated with β-cell regeneration and trans-differentiation (Jamal et al., 2005; Pittenger et al., 2007). We observed induction of the *REG3A* gene expression in samples of non-endocrine pancreatic cells upon treatment with LIF. Co-administration of LIF with nicotinamide and dexamethasone even further increased expression of the *REG3A* gene. This is in an agreement with previous reports that showed positive effect of the LIF/IL-6 cytokine family on the expression of REG3A and INGAP proteins (Nata et al., 2004; Taylor-Fishwick et al., 2006). The positive effect of REG3A/INGAP proteins on β-cell differentiation can be explained by a stimulatory effect of these proteins on the expression of PDX1 transcription factor (Rosenberg et al., 2004). PDX1 is not only involved in β-cell differentiation, but also stimulates insulin gene expression (Shao et al., 2009). In our study, induction of *REG3A* expression was followed by stimulation of *PDX1* expression. In contrast, expression of the *PDX1* gene was significantly lower in control cells not treated with LIF. The lower expression of *PDX1* may be attributed to the absence of *REG3A* expression in control samples.

Treatment of pancreatic non-endocrine cells with LIF not only induced expression of the REG3A protein and transcription factor PDX1, but also stimulated expression of neurogenin 3, one of the key transcription factors of pancreatic endocrine cell differentiation. This result is also in accordance with the previous report showing that treatment of dedifferentiated pancreatic exocrine cells with LIF and EGF induces transient expression of neurogenin 3 and its upstream activator hepatocyte nuclear factor 6 (HNF6) (Baeyens et al., 2006). The authors assume that the up-regulation of HNF6 transcription factor upon treatment with LIF and EGF leads to the expression of the neurogenin 3 gene. However, in our study we have revealed that the expression of neurogenin 3 induced by LIF may also be attributed to the effect of LIF on the Notch signalling pathway. The expression of neurogenin 3 is known to be repressed by activation of the Notch signaling pathway (Murtaugh et al., 2003). The inhibitory effect of the Notch signalling pathway is mediated by the HES1 transcription factor, a downstream effector of the Notch pathway (Kageyama et al., 2007). Promoter of the neurogenin 3 gene contains multiple binding sites for HES1, which acts as a repressor of neurogenin 3 expression (Lee et al., 2001). Therefore, activation of the *HES1* gene expression by the Notch signalling pathway leads to the inhibition of neurogenin 3 gene expression.

In our study, we detected stable expression of the *HES1* gene in all samples during the entire differentiation period. *HES1* was also expressed by the non-endocrine pancreatic cell population obtained from islet-depleted pancreatic tissue prior to differentiation. In addition to the *HES1* gene expression we also evaluated expression of the *HES6* gene. HES6 acts as a suppressor of the Notch signalling pathway by inhibiting the interaction of HES1 with its transcriptional co-repressor Gro/TLE. Moreover, HES6 also promotes proteolytic degradation of HES1 (Gratton et al., 2003). In our study, we detected significantly higher expression of the *HES6* gene in the samples treated with LIF than in the control sample. Upon removal of LIF and further differentiation the level of *HES6* expression decreased; however, it was still higher in samples previously treated with LIF than in the control cells. Based on these results we assume that the positive effect of LIF on neurogenin 3 expression may be explained by two mechanisms. Firstly, LIF stimulates the expression of *HNF6*, an upstream activator of neurogenin 3 expression (Zhang et al., 2009). In addition, the induction of *HES6* expression by LIF further stimulates neurogenin 3 expression by repressing the inhibitory effect of HES1.

In conclusion, we report here that activation of the Jak/Stat signalling pathway stimulates differentiation of human non-endocrine pancreatic cells into insulin-producing β-cells. The positive effect of LIF treatment on β-cell differentiation may be attributed to the activation of PDX1 and neurogenin 3 expression, two of the key transcription factors of β-cell differentiation. The stimulatory effect of LIF is most likely indirect. In the case of neurogenin 3 gene expression the stimulatory effect of LIF seems to be mediated by the transcription factor HNF6 and the suppressor of Notch signalling pathway HES6, while the positive effect of LIF on PDX1 up-regulation seems to be promoted by induction of the *REG3A* gene expression.

The Jak/Stat signalling pathway plays an important role in differentiation of neural precursor cells during embryonic development and postnatal life. Pancreatic endocrine cells and neurons share a lot of common transcription factors and regulatory mechanisms that control their differentiation (Atouf et al., 1997; Apelqvist et al., 1999; van Arensbergen et al., 2010). It is therefore not surprising that the Jak/Stat signalling pathway also plays an important role in differentiation of neurons as well as pancreatic β-cells. Our results support previous reports about the positive effect of the Jak/Stat signalling pathway activation on trans-differentiation of insulin-producing cells and uncover underlying interactions between the Jak/Stat and Notch signalling pathways.

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Original Article

In Vivo Differentiation of Human Umbilical Cord Blood-Derived Cells into Insulin-Producing β Cells

(diabetes mellitus / insulin / pancreas / β cell / islets / stem cells / umbilical cord blood / differentiation / *in vivo* / radiation/transplantation)

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Abstract. In our study we confirmed the potential of human umbilical cord blood cells to differentiate into insulin-producing cells following transplantation into immunocompromised mice. The average number of C-peptide-positive human cells per animal was 18 ± 13 as assessed by immunofluorescence staining and fluorescence *in situ* hybridization specific for human ALU sequence. Differentiation into insulin-producing cells was further confirmed by reverse transcription-polymerase chain reaction specific for human insulin mRNA. Successful differentiation required sublethal irradiation of xenogeneic recipient at least at a dose of 3 Gy. However, transplantation of human umbilical cord blood cells did not improve hyperglycaemia in diabetic animals. The results of our study show that human umbilical cord blood may be considered as a potential source of stem cells for treatment of diabetes mellitus.

Introduction

Despite advances in the treatment of diabetic patients, diabetes remains one of the most serious health care problems of our civilization. Clinical islets or pancreas transplantations are the only available therapies able to establish insulin independence and long-lasting normoglycaemia (Shapiro et al., 2000; White et al., 2009). However, the lack of donors limits the application of this therapy for all type 1 diabetic patients in need.

The discovery of stem cells and their successful differentiation into insulin-producing β cells gave a new hope to all diabetic patients. Within last few years various types of stem cells able to give rise to the pancreatic β cells have been identified. They include embryonic and foetal stem cells (Zhang et al., 2005; D'Amour et al., 2006) as well as adult stem cells derived from pancreas, liver, bone marrow and central nervous system (Bonner-Weir et al., 2000; Yang et al., 2002; Ianus et al., 2003).

In addition to these "traditional" sources of adult stem cells, umbilical cord blood-derived stem cells have emerged as a new potential source for cell-based therapies. The main advantages of human umbilical cord blood (HUCB) include plentiful availability, safe and non-invasive procedure of collection, possible expansion and modification of cells *in vitro* and an existing network of umbilical cord blood banks, a large-scale source of cells that allows matching the donor and host human leukocyte antigen (HLA) systems. HUCB, highly enriched for haematopoietic stem cells, has already been successfully applied for the treatment of various blood diseases (Roche et al., 2000; Laughlin et al., 2004). Moreover, several recent reports have shown that some of the HUCB cells are able to differentiate into multiple cell types of non-haematopoietic origin (Kogler et al., 2004; McGuckin et al., 2005). These findings suggest that umbilical cord blood contains multipotent stem cells or primitive progenitors that might have the potential to differentiate into cells of non-haematopoietic phenotype, including pancreatic β cells.

Denner as the first demonstrated successful *in vitro* differentiation of HUCB stem cells into insulin- and C-peptide-producing cells (Denner et al., 2007). Two other groups lately reported similar results using differ-

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Abbreviations: DAPI – 4,6-diamidino-2-phenylindole, EBSS – Earle's balanced salt solution, FISH – fluorescence *in situ* hybridization, HLA – human leukocyte antigen, HUCB – human umbilical cord blood, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, MNCs – mononuclear cells, NK – natural killer, NOD/SCID – non-obese diabetic/severe combined immunodeficient/β_2-microglobulin null mice, PCR – polymerase chain reaction, RT-PCR – reverse transcriptase polymerase chain reaction.

ent approaches (Sun et al., 2007; Gao et al., 2008). Sun's group used a specific subpopulation of HUCB cells expressing embryonic markers Oct-4 and SSEA-4. These cells were differentiated by a protocol using only nicotinamide and extracellular matrix proteins laminin and fibronectin. Gao's group worked with HUCB-derived mesenchymal stem cells and employed a more complicated protocol including retinoic acid, nicotinamide, exendin-4 and extracellular matrix proteins. In spite of successful differentiation into insulin-producing cells, the secretion of insulin in response to increased glucose levels was not significantly higher than that at basal conditions. This phenomenon is quite common in case of *in vitro* derived β cells and may be explained by immaturity of this cell type (D'Amour et al., 2006).

In vivo differentiation of HUCB cells into the pancreatic β cells has so far been demonstrated only by Yoshida et al. The presence of human insulin-producing cells in mouse pancreatic tissue after transplantation of T cell-depleted HUCB mononuclear cells (MNCs) into newborn non-obese diabetic/severe combined immunodeficient mice have been reported by the authors. However, the average number of HUCB-derived insulin-producing cells per total number of islet cells was only 0.65 %. The low rate of human β cells within mouse pancreas could be explained by a non-diabetic status of the animals (Yoshida et al., 2005). Under diabetic conditions, the demand for neogenesis of insulin-producing cells might be increased and the higher rate of HUCB cell differentiation might represent a compensatory effect in face to a decreased β-cell mass.

In light of these results we decided to investigate the conditions that stimulate *in vivo* differentiation of HUCB mononuclear cells into insulin-producing cells. Survival, homing and differentiation of HUCB cells were studied in athymic nude mice, which do not reject xenografts and thus represent a suitable model for transplantation of human cells. We tested the effect of the whole body irradiation, which had been shown to increase homing and engraftment of human cells in transplanted mice (Becker et al., 2002). Finally, we also examined the possibility to treat the streptozotocin-induced diabetes by transplantation and possible differentiation of HUCB cells into insulin-producing cells.

Here we report that HUCB-derived mononuclear cells convincingly do have the potential to differentiate into a β cell-like phenotype, though, with the use of current protocols, only at a very low rate that still does not reach a therapeutic significance.

Material and Methods

Study design

For the purpose of our study mice were divided into the groups based on the applied radiation dose and eventual induction of diabetes (Table 1). All animals with the exception of the control group were injected with 10^7 unpurified HUCB mononuclear cells into the tail vein. Mice in groups 2, 3, 5 and 6 underwent total body irradiation one day prior to the application of HUCB cells at the dose of 1 (groups 2 and 5) or 3 Gy (groups 3 and 6). In groups 4, 5 and 6 diabetes was induced by streptozotocin three days prior to the application of HUCB cells. In diabetic animals, fed blood glucose was monitored at weekly intervals during the experiment. Animals were sacrificed at the end of 4th week and tissue samples were collected for further analysis.

Isolation of HUCB Cells

Samples of HUCB (40–120 ml) were obtained at the end of physiological delivery. At the admission to hospital, all donors signed an informed consent approved by the Institutional Ethical Committee of the Institute for Clinical and Experimental Medicine and Thomayer Teaching Hospital. Samples of HUCB were collected into standard blood donor bags containing 15 ml of citrate phosphate dextrose (Baxter Healthcare, Deerfield, IL). HUCB was diluted in a ratio 1 : 2 with Earle's balanced salt solution (EBSS) (Sigma-Aldrich, Steinheim, Germany) and centrifuged at 400 *g* for 20 min at 4 °C on a layer of Ficoll-Hypaque 1.077 (Sigma-Aldrich). Mononuclear cells (MNCs) at the interface of supernatant were washed twice with EBSS. Viability was determined by the trypan blue dye exclusion method. MNCs were resuspended in Iscove's modified Dulbecco's medium (Sigma-Aldrich) containing 20% foetal bovine serum (Biochrom, Berlin, Germany) and cryopreserved with 10% (vol/vol) DMSO (Sigma-Aldrich).

Mice

Female nude athymic mice (Crl:CD1-nu strain, AnLab, Prague, Czech Republic) were maintained under defined flora with irradiated food and sterile water in sterile cages at the animal facility. All experiments were approved by the Committee for Animal Ethical Treatment of the Institute for Clinical and Experimental Medicine.

Table 1. Groups of animals based on the radiation dose and diabetes induction

	Radiation dose (Gy)	Induction of diabetes	HUCB transplantation
Group 1	0	No	Yes
Group 2	1	No	Yes
Group 3	3	No	Yes
Group 4	0	Yes	Yes
Group 5	1	Yes	Yes
Group 6	3	Yes	Yes
Group 7 (control group)	0	No	No

Induction of diabetes

Athymic mice aged 6–8 weeks were treated with a single intravenous dose of 250 mg/kg streptozotocin (Sigma-Aldrich) freshly dissolved in citrate buffer (0.05 mM, pH 4.5). Mice were considered as diabetic when non-fasting blood glucose levels were > 16 mmol/l on three consecutive days.

HUCB Transplantation

Prior to transplantation, cryopreserved HUCB MNCs were thawed, counted and tested for viability by the trypan blue dye exclusion method. The amount of 10^7 MNCs was injected intravenously into the tail vein of non-diabetic or diabetic mice (8 weeks old). Two groups of diabetic and two groups of non-diabetic mice were conditioned with 1 or 3 Gy of total body irradiation one day prior to the transplantation.

Pancreatic Islet Isolation

Mouse pancreatic islets from the HUCB cell recipients and control animals were isolated using the collagenase digestion method as previously described (Berkova et al., 2005). The pancreases were injected with 1 ml of collagenase at a concentration of 2 mg/ml (Sevapharma, Prague, Czech Republic) and incubated at 37 °C for 15 min in a total of 10 ml of digestion solution under constant shaking. Islets were subsequently washed three times in Hank's balanced salt solution (HBSS) (Biochrom) with bovine serum albumin (BSA) (5 mg/ml) and purified with the use of discontinuous gradients of Ficoll-diatrizoic acid (Sigma-Aldrich). The solution densities of discontinuous Ficoll gradient ranged from 1.034 to 1.1162 g/ml with the densest solution at the bottom of density gradient. During centrifugation, islets migrated to the interface between 1.070 and 1.080 g/ml. The remaining cellular material from the denser layer was also pooled and further processed for gene expression analysis.

FISH and Immunofluorescence Analysis

After the pancreatic tissues were harvested from the recipient mice, the tissues were fixed with Bouin's solution (Sigma-Aldrich) for 2 h at room temperature. The tissues were rinsed with PBS, embedded in OCT mounting medium (Bayer Corp, Pittsburgh, PA), frozen in liquid nitrogen, and stored at -80 °C.

After several washes in PBS, 8-μm sections of frozen tissue were incubated in a solution containing 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 20 min. Antigen retrieval method for immunofluorescence staining was performed prior to fluorescence *in situ* hybridization (FISH). Slides were heated twice in 0.01 M sodium citrate (Sigma-Aldrich), pH 6.0, in a microwave oven for periods of 4 min at the maximal power setting (900 W) with 120–140 s of boiling. Slides were incubated in blocking solution containing 5% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA). After dehydratation in 70%, 90% and 100% ethanol for 2 min each, slides were incubated in 50% formamide/2× SSC denaturing solution for 5 min at 75 °C. After denaturation, the slides were incubated overnight at 37 °C with Alexa555-conjugated ALU-species-specific probe diluted in hybridization buffer (50 ng/100 ul). An Alu-specific probe binds the ALU-sequence that is present only in primate genomes. Alu sequences are about 300 base pairs long and form about 10 % of the human genome. The sequence of the Alu-specific probe is given in Table 2.

After the hybridization, slides were washed three times in 50% formamide/2× SSC for 5 min each at 42 °C. For immunofluorescence co-staining, slides were blocked in 5% donkey serum (Jackson Immunoresearch Laboratories) diluted in 0.2% Triton X-100, 0.1 M glycine (Sigma-Aldrich) and PBS for 1 h at room temperature to prevent unspecific binding. Incubation with rabbit anti-human C-peptide antibody (Linco-Research, St. Charles, MO) diluted 1 : 200 was performed in the same blocking solution for 1 h at 37 °C. After intensive washing with PBS, slides were incubated with the secondary antibody Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probe, Eugene, OR) diluted in the blocking solution for 1 h at 37 °C. 4,6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at a concentration 5 μg/ml was used to label the nuclei for 10 min at 37 °C. After rinsing with PBS, sections were mounted with antifade solution and examined with a fluorescence microscope (BX 41, Olympus, Tokyo, Japan).

Reverse Transcription-Polymerase Chain Reaction

RNA was isolated from pancreatic islets and remaining pancreatic tissue of the recipient mice using Rneasy Plus Mini Kit (Qiagen, Hilden, Germany). Isolated RNA was treated with DNAse using RNase-Free DNase Set

Table 2. Primers and Alu-specific probe sequences used for the RT-PCR and FISH analysis

Primer	sequence	product size (bp)	annealing temp. (°C)	cycle number
human insulin forward	agccgcagcctttgtgaac	141	63	45
human insulin reverse	agctccacctgccccac	141	63	45
mouse insulin forward	ctataatcagagaccatcagcaagc	344	60	35
mouse insulin reverse	gtagagggagcagatgctgg	344	60	35
human GAPDH forward	gagtcaacggatttggtcg	141	59	40
human GAPDH reverse	catgggtggaatcatattgg	141	59	40
Alu-specific probe	cctgtaatcccagctactcgggagg ctgaggcaggagaatcgcttgaacc		37	

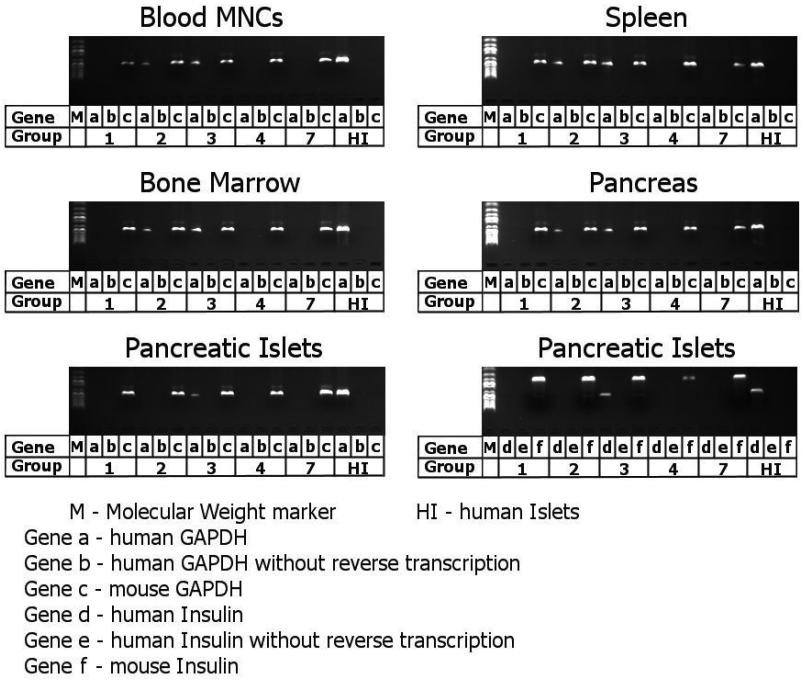

Fig. 1. RT-PCR analysis of gene expression in mouse tissues. Transcripts of human *GAPDH* (a) and insulin (d) were analysed and compared with trancripts of mouse *GAPDH* (c) and insulin genes (f). The products of PCR reaction without reverse transcription served as a negative control (b, e). Human islet RNA was used as a positive control.

(Qiagen) and 1 μg of RNA was reverse transcribed with Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. cDNA was amplified using HotStarTaq Master Mix Kit (Qiagen). Gene-specific primer pairs, annealing temperatures, and product sizes are listed in Table 2. All of the primers span at least one of the introns to prevent false-positive results. PCR products were separated and visualized on 2% agarose gel containing ethidium bromide.

Results

Successful HUCB transplantation was confirmed by detection of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in samples isolated from mouse blood MNCs 4 h after the transplantation (Fig. 1). Further detection of human cells within mouse tissues was performed four weeks after the application of HUCB cells using the primers specific for human GAPDH. The expression of human GAPDH was detected only in samples derived from mice exposed to both 1 and 3 Gy radiation doses. Human GAPDH was detected in all of the examined tissues (spleen, bone-marrow, blood MNCs and pancreatic tissue). No expression of human GAPDH was detected in tissue samples from mice that had not been subjected to total body irradiation (Table 3, Fig. 1).

In order to evaluate the potential of HUCB-derived cells to differentiate into human insulin-producing cells we performed RT-PCR analysis of human insulin gene expression. We used RNA isolated from fresh pancreatic tissue and Langerhans islets of the recipient mice. The expression of human insulin mRNA was observed exclusively in pancreatic tissue derived from mice irradiated with 3 Gy. We did not detect any expression of human insulin gene either in the isolated islets or pancreatic tissue derived from mice irradiated with only 1 Gy or in samples from unirradiated mice. For the detection of human insulin mRNA, we designed the forward and reverse primers that specifically amplified human but not mouse insulin cDNA or human gDNA. The amplified products derived from the recipients' pancreata were clearly seen on agarose gel (Fig. 1). The product size corresponded to the expected size of PCR reaction product amplified by specific primers. These results indicate that human insulin was produced by donor

Table 3. Detection of human cells within examined mouse tissues in the study groups

Tissue	Blood	Spleen	Bone Marrow	Pancreas	Islets	Human β cells
Group 1	-	-	-	-	-	-
Group 2	+	+	+	+	-	-
Group 3	+	+	+	+	+	+
Group 4	-	-	-	-	-	-
Group 5	N/A	N/A	N/A	N/A	N/A	N/A
Group 6	N/A	N/A	N/A	N/A	N/A	N/A
Group 7	-	-	-	-	-	-

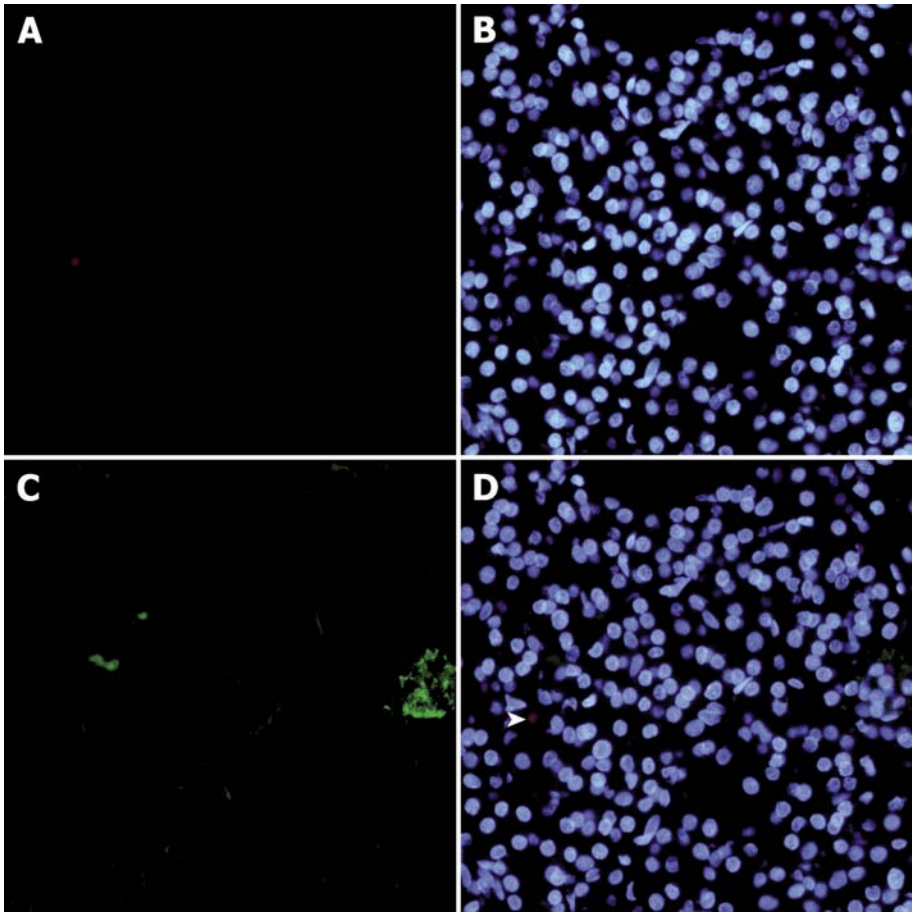

Fig. 2. FISH and immunofluorescence staining of mouse pancreatic tissue four weeks after the irradiation with 1 Gy. (A) FISH for human ALU sequence (red), (B) DAPI staining of nuclei (blue), (C) C-peptide immunofluorescence staining (green) and (D) merged images A–C. HUCB-derived ALU sequence-positive cell is shown (arrowhead).

HUCB-derived cells in the recipient pancreas at the RNA level. Insulin-specific cDNA in the tissue obviously did not originate from passenger haematopoietic cells as this reaction was negative in all starting MNC samples.

The presence of human cells within pancreatic tissue was further confirmed by the fluorescence *in situ* hybridization specific for the human ALU sequence. Rare human cells were detected within the acinar tissue, islets and also in the pancreatic ducts of recipient mice (Fig. 2 and Fig. 3).

In the next step we performed immunofluorescence labelling of human C-peptide in combination with human ALU sequence-specific FISH to confirm the expression of human insulin at the protein level. C-peptide-positive human cells were clearly demonstrated within the pancreatic islets of mice irradiated with 3 Gy, although only in a low number (Fig. 3). The average number of C-peptide-positive human cells per animal was 18 ± 13. The whole pancreata were cut into 10-µm sections and the investigation was performed in all of them.

Transplantation of HUCB into severely diabetic animals did not lead to metabolic improvement. All seven animals progressively wasted and died before the end of the study period. Therefore, the presence of human cells within their tissues could not be studied.

Discussion

For their availability and easy storage, umbilical cord stem or precursor cells have been regarded as a promising source for cellular therapy of diabetes, though the scientific and practical reasons for this hope have still been lacking. The ability to differentiate into the β-cell phenotype undoubtedly depends on selection of the right cell type, on its culture conditions and last but not least, on the post-transplant care of the recipient. Cure or significant improvement of experimental diabetes by HUBC transplantation has not been achieved in any study so far. However, the results of our study demonstrated that the potential of HUCB mononuclear cells to engraft in the host pancreas and to differentiate into insulin-producing cells does exist. The origin of the transplanted cells was confirmed not only by fluorescence *in situ* hybridization staining for specific human DNA sequence in combination with immunofluorescence staining for human insulin in transplanted immunocompromised mice, but also by highly sensitive RT-PCR detection of human insulin mRNA.

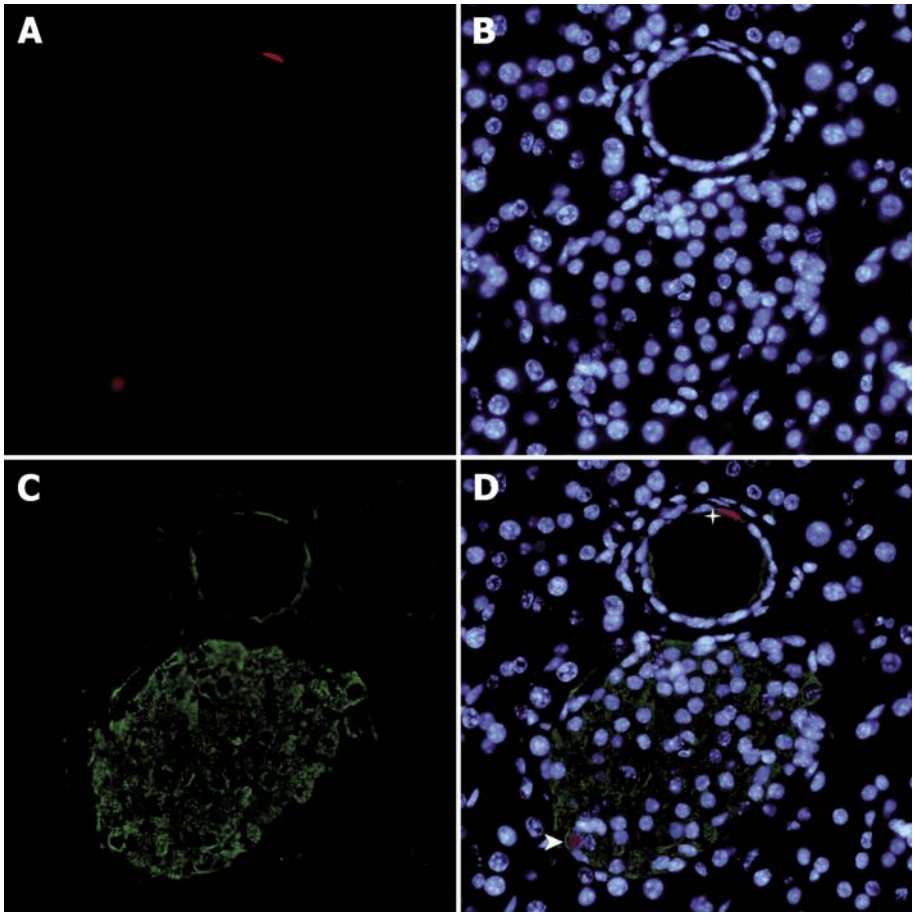

Fig. 3. FISH and immunofluorescence staining of mouse pancreatic tissue four weeks after the irradiation with 3 Gy. (A) FISH for human ALU sequence (red), (B) DAPI staining of nuclei (blue), (C) C-peptide immunofluorescence staining (green) and (D) merged images A–C. HUCB-derived ALU sequence and C-peptide-positive cell in pancreatic islet is shown (arrowhead) and HUCB-derived ALU sequence-positive cell in pancreatic duct is shown (star).

Our results are in agreement with those published by Yoshida's group (Yoshida et al., 2005). They also observed the presence of human insulin-producing cells in pancreatic tissue after the transplantation of HUCB mononuclear cells into the normoglycaemic mice. In their experiment, the rate of differentiation was significantly higher in comparison with our study. The difference between the numbers of differentiated human β cells may be due to different mouse strains used in the studies as well as the type and number of transplanted cells. Yoshida et al. (2005) used non-obese diabetic/severe combined immunodeficient/β_2-microglobulin null mice (NOD/SCID/β2m null), which lack mature T as well as B cells and show extremely low activity of natural killer (NK) cells. This profound immunological incompetence obviously enables high engraftment rates of human cells in NOD/SCID/β_2m null mice (Ishikawa et al., 2002). In our study we used the CD-1-nu/nu nude mouse strain, which lacks only mature T lymphocytes but still shows functional antibody-producing B and NK cells. The nude mouse strain may provide a lower engraftment potential for xenografts in comparison with the NOD/SCID strain as demonstrated by transplantation of foetal porcine pancreatic tissue into the NOD/SCID and nude mice (Tuch et al., 1999).

We have therefore decided to use whole-body irradiation in the effort to increase engraftment efficiency of human cells. Without irradiation pretreatment we found neither any human insulin-producing cells nor any human cells in pancreatic or any other examined tissues. Conversely, after a 1 Gy total body irradiation we detected expression of human GAPDH in haematopoietic organs such as spleen, blood and bone marrow using PCR detection. Nevertheless, we did not detect any expression of human insulin gene in pancreatic tissue. Further increase of the radiation dose up to 3 Gy led not only to the engraftment of human cells in pancreatic tissue but also allowed differentiation of human cells into insulin-producing cells. While most of the human cells within the host pancreatic tissue were insulin-negative, we convincingly detected a few insulin-positive human cells in the pancreatic islets. Noteworthy is also the presence of human cells in pancreatic ducts. Since islet cell neogenesis is thought to occur in pancreatic ducts (Slack, 1995), it could be speculated that human cells present in pancreatic ducts may undergo differentiation

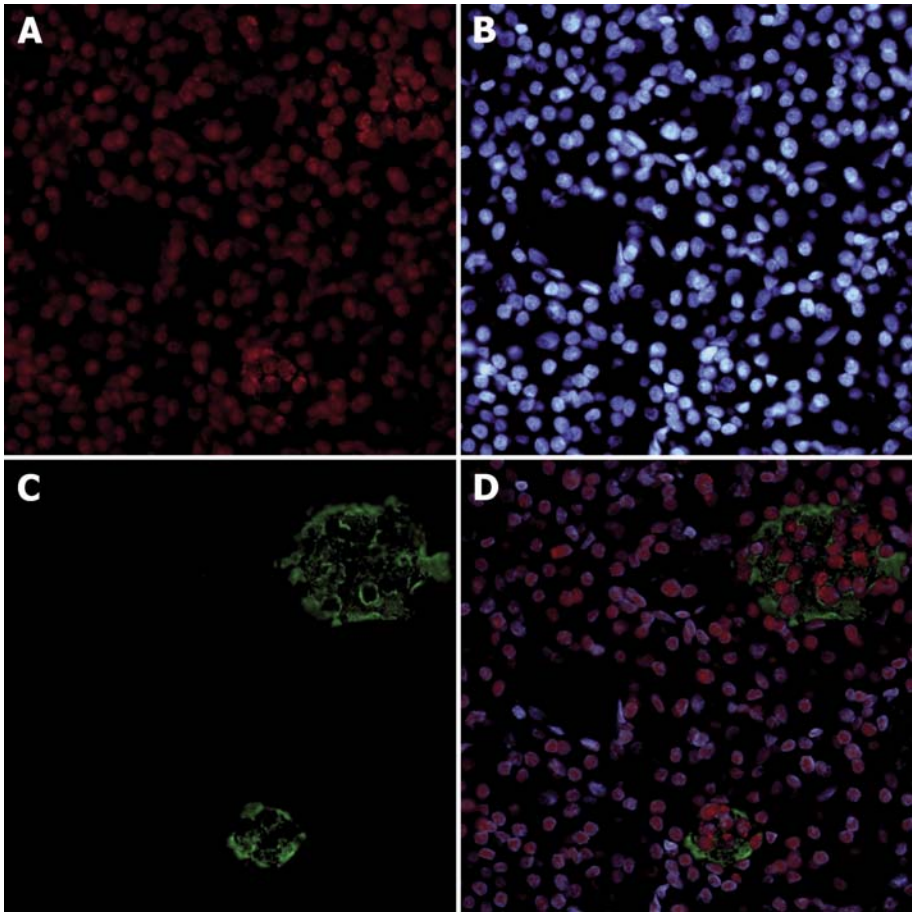

Fig. 4. FISH and immunofluorescence staining of human pancreatic tissue. (A) FISH for human ALU sequence (red), (B) DAPI staining of nuclei (blue), (C) C-peptide immunofluorescence staining (green) and (D) merged images A–C.

into the endocrine cells under the influence of pancreatic ductal niche.

In contrast to the paper of Yoshida et al. (2005), the presence of human insulin-producing cells in the host pancreas and in isolated pancreatic islets was undoubtedly confirmed by specific PCR detection of human insulin mRNA. The lower number of human insulin-positive cells in our study may also be explained by the lower number of transplanted cells. Isolation of HUCB cells from one donor enabled transplantation of 10^7 MNC into 5–10 mouse recipients. Yoshida et al. reported application of 10^7 $CD3^+$, $CD4^+$ and $CD8^+$-depleted MNCs, which represents approximately 35 % of all HUCB MNCs (Pranke et al., 2001). Therefore, we assume that for one mouse recipient they had to use a higher amount of HUCB than we used.

The explanation for successful engraftment and differentiation of HUCB cells in pancreatic tissue after the radiation treatment is not evident from our results. One could speculate that the tissue damage caused by radiation stimulates migration and engraftment of human stem cells into the injured organs. For example, depletion of the host immune system and haematopoietic stem cell pool by radiation-mediated myeloablation led to successful engraftment of donor stem cells in haematopoietic organs (Stewart et al., 1998). The positive effect of radiation on the engraftment of stem cells and tissue regeneration is not characteristic only for haematopoietic tissue, but also for neural (Marshall et al., 2005) and hepatic tissues (Guha et al., 2001).

Another condition which could have allowed engraftment of human cells is the radiation-mediated myeloablation of the mouse immune system. Depletion of host immune cells caused by myeloablation may have impaired xenograft rejection mediated by the remaining B and NK cells (Yoshino et al., 2000). Although we have no direct evidence how severe the depletion of the mouse immune system caused by irradiation was, we suppose that increasing doses of radiation allowed higher engraftment rate of HUCB cells into haematopoietic tissue with consequent restoration of the impaired immune system. A rather high prevalence of GAPDH-positive cells that we found in the peripheral blood and bone marrow supports this assumption.

An important stimulus for stem cell differentiation into insulin-producing cells might be the diabetes-related hyperglycaemia (Wang et al., 2005). In our study, HUCB administration did not cure or improve streptozotocin-induced experimental diabetes. Previous study conducted by Ende et al. has shown improvement in glycaemia and survival of diabetic mice after the transplantation of HUCB cells (Ende et al., 2004). However,

in their study the dose of HUCB cells was 200 × 10^6, 20-fold higher than in our study. In this paper, however, no investigation of insulin-positive human cells had been performed. For further study, a longer time period and milder hyperglycaemia enabling survival will be necessary.

In conclusion, our study confirmed the possibility of human umbilical cord blood mononuclear cells to differentiate into human insulin-producing cells *in vivo*. However, successful differentiation occurred at a rather low rate and required preceding irradiation of the immunodeficient mouse recipient. Further investigation should focus on other potential conditions that might stimulate β-cell differentiation *in vivo* such as hyperglycaemia, administration of incretins, and on identification of the appropriate umbilical cord blood cell type suitable for transplantation.

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