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.

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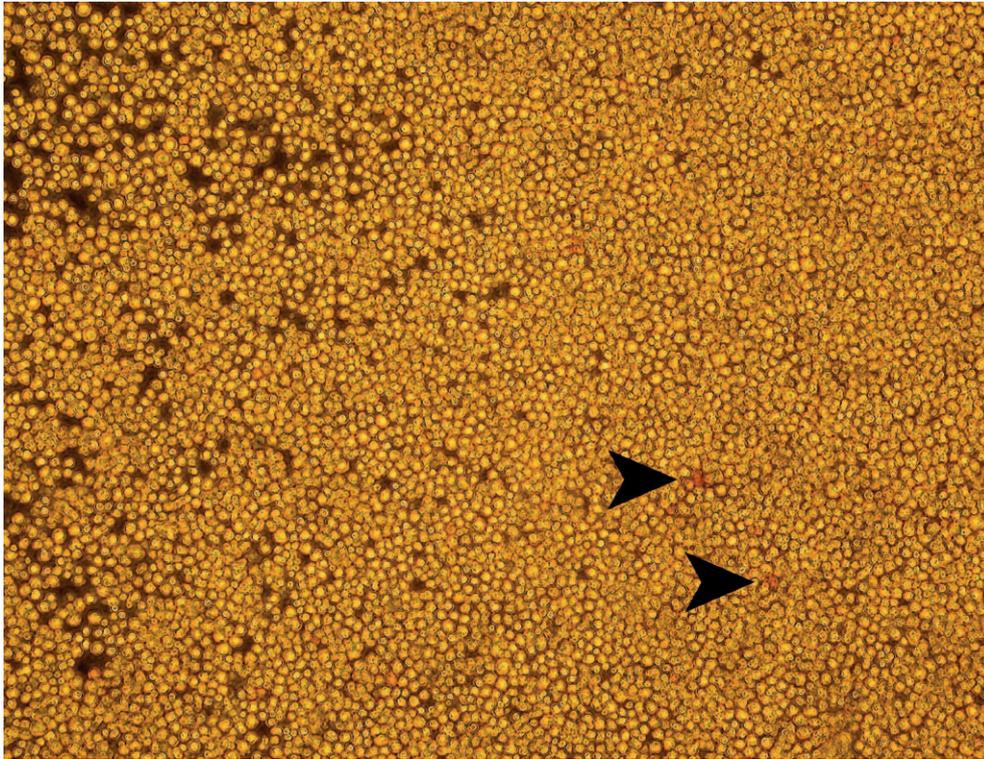
Fig. 1. Dithizone staining of cell suspension derived from dissociated islet-depleted pancreatic tissue. Red dithizone-stained insulin-positive cells (arrows).
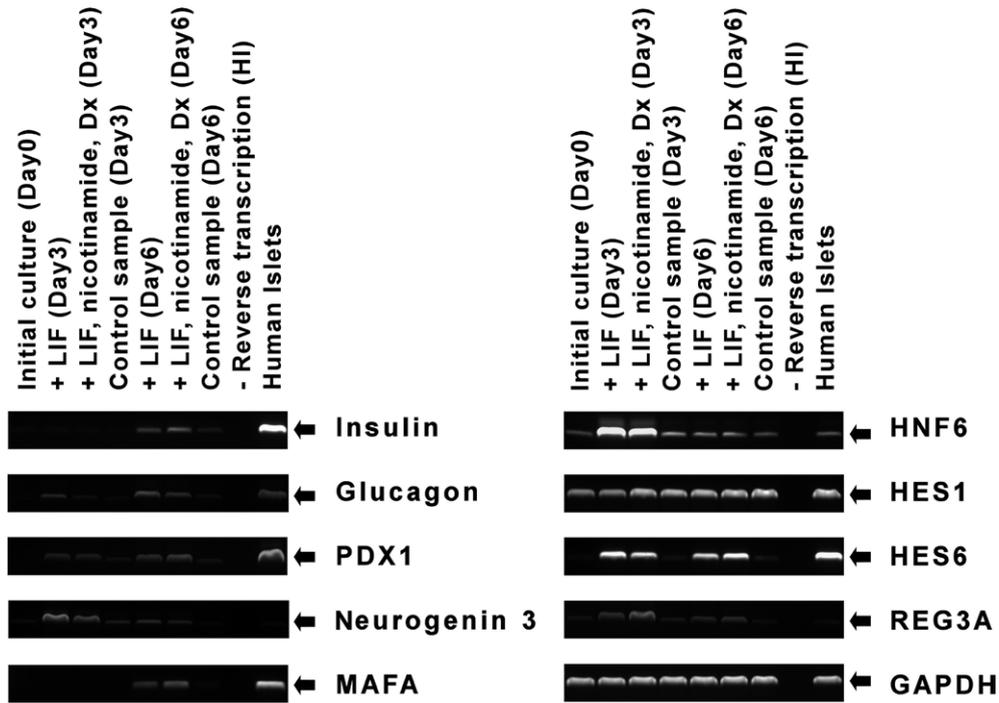

Fig. 2. Reverse transcriptase polymerase chain reaction analysis of gene expression during differentiation of pancreatic non-endocrine cells. RNA isolated from human pancreatic islets was used as a positive control. RNA isolated from human pancreatic islets without reverse transcription was used as a 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
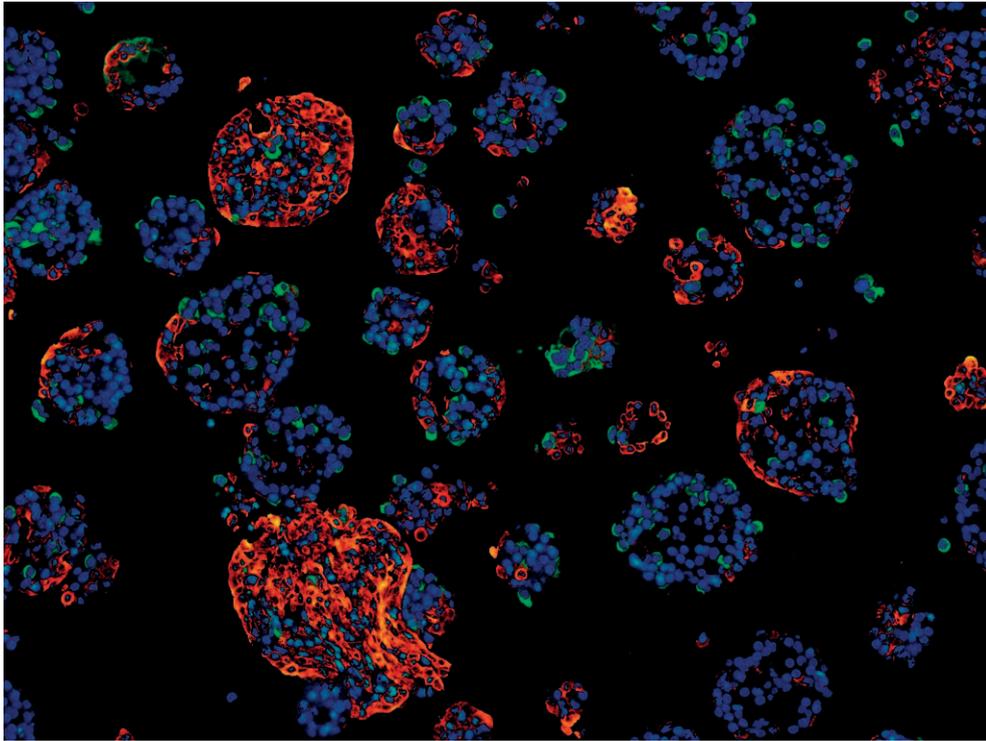
Fig. 3A. Immunofluorescence staining of cytokeratin-19 (orange) and C-peptide (green) in ILCCs treated with a combination of LIF, nicotinamide and dexamethasone during the first three days of the culture period. DAPI (blue) stain was performed as counter stain (magnification 100×).
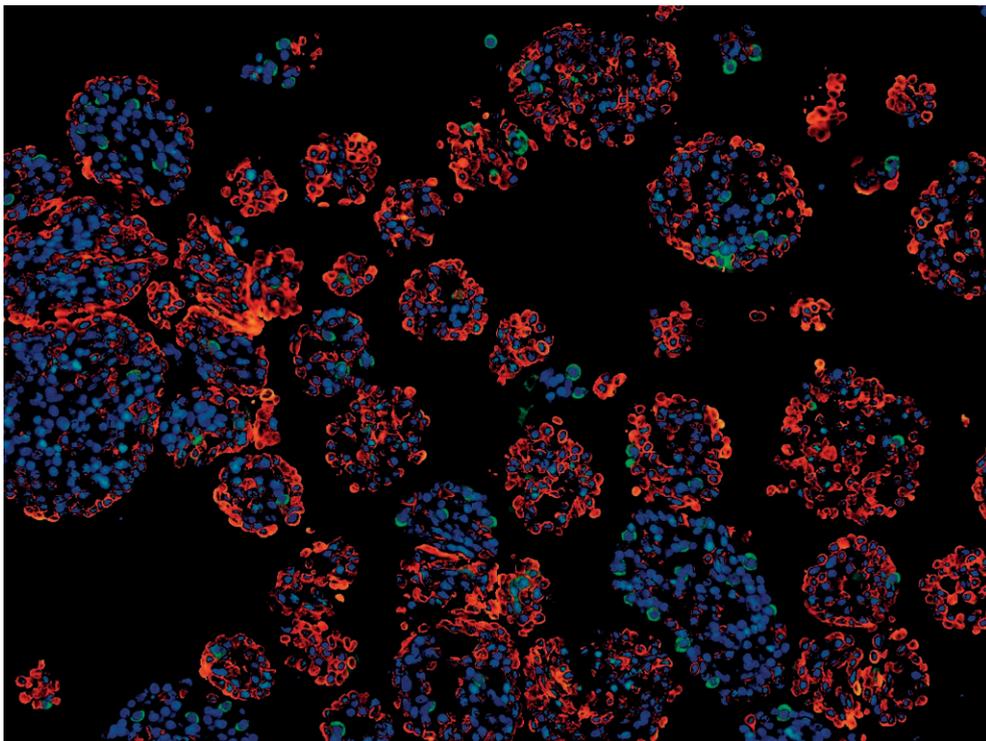

Fig. 3B. Immunofluorescence staining of cytokeratin-19 (orange) and C-peptide (green) in ILCCs treated with LIF during the first three days of the culture period. DAPI (blue) stain was performed as counter stain (magnification 100×).

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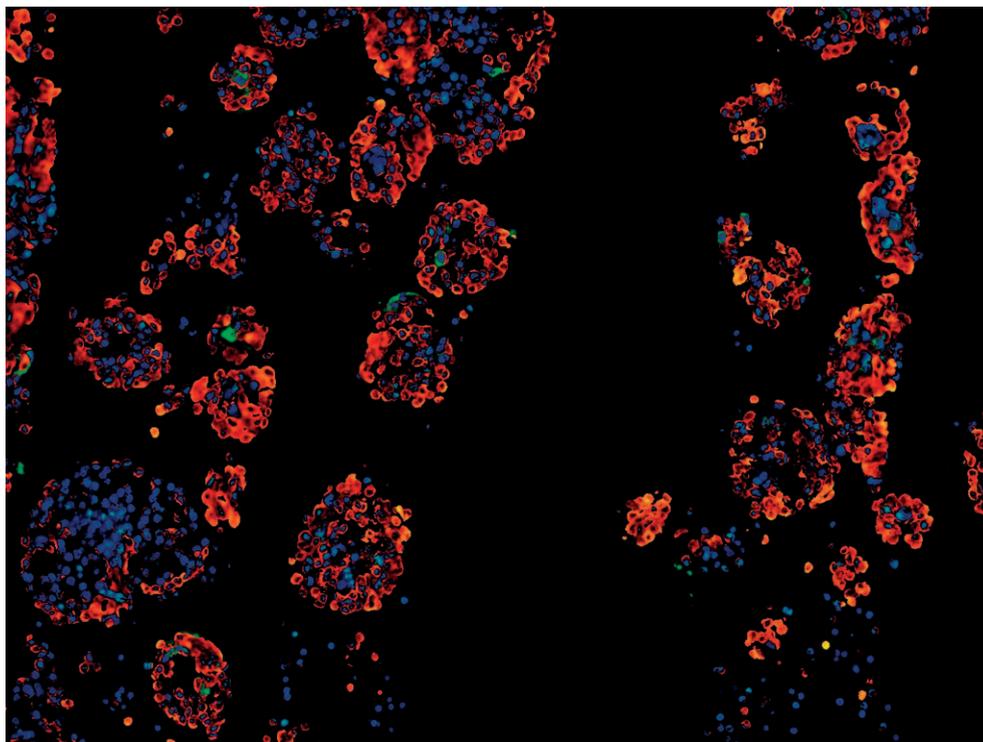
Fig. 3C. Immunofluorescence staining of cytokerratin-19 (orange) and C-peptide (green) in ILCC control sample untreated with any of the tested compounds during the first three days of the culture period. DAPI (blue) stain was performed as counter stain (magnification 100×).

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.
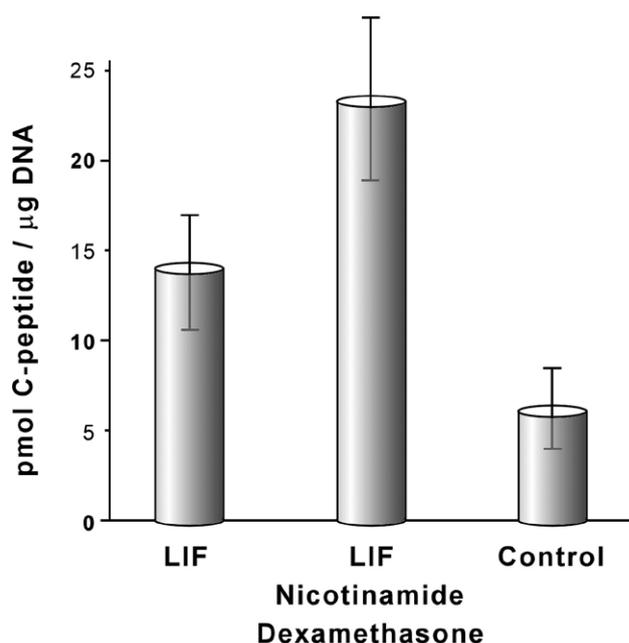

Fig. 4. C-peptide content of non-endocrine pancreatic cells treated with LIF or with a combination of LIF, nicotinamide and dexamethasone and control sample as determined by IRMA.

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 con-

tains 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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