Isolation and Characterization of Human CXCR4-Positive Pancreatic Cells

(diabetes mellitus / insulin / pancreas / β cell / islets / stem cells / CXCR4)

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Abstract. The existence of an adult PSC that may be used in the treatment of diabetes is still a matter of scientific debate as conclusive evidence of such a stem cell in the adult pancreas has not yet been presented. The main reason why putative PSC has not yet been identified is the lack of specific markers that may be used to isolate and purify them. In order to increase the list of potential PSC markers we have focused on the human pancreatic cells that express cell surface receptor CXCR4, a marker of stem cells derived from different adult tissues. Here we report that CXCR4-positive pancreatic cells express markers of pancreatic endocrine progenitors (neurogenin-3, nestin) and markers of pluripotent stem cells (Oct-4, Nanog, ABCG2, CD133, CD117). Upon *in vitro* differentiation, these cells form ILCC and produce key islet hormones including insulin. Based on our results, we assume that CXCR4 marks pancreatic endocrine progenitors and in combination with other cell surface markers may be used in the attempt to identify and isolate PSC.

Diabetes mellitus (DM) is a chronic, life-threatening disease characterized by a metabolic disorder in which there is a lack of action or production of insulin. Type 1 DM is caused by an autoimmune reaction leading to the destruction of the insulin-producing β-cells in the pancreas. Current methods of DM treatment are not capable to achieve tight control of blood sugar level resulting in serious long-term complications, including eye, kidney, nerve and heart disease. Presently, the only approaches to achieving normal glucose control are transplantation of the whole pancreas or transplantation of isolated pancreatic islets (Shapiro et al., 2000; Robertson et al., 2006). This kind of treatment is currently available only for a highly selected group of patients. Whole organ pancreas transplantation is usually performed in diabetic patients with kidney failure who are simultaneously undergoing kidney transplantation (Robertson et al., 2006). A major obstacle to providing whole pancreas or islet transplantation to those in need is the lack of organs as they can only be obtained from cadaver donors. Therefore, the research in the diabetes field has focused on the identification of new sources of insulin-producing tissue.

The embryonic or adult stem cells are considered to be the most promising source of such tissue. Embryonic stem cells (ESC) may seem to be the best material for generating new β-cells as they are pluripotent and can differentiate into any cell of three germ layers including endoderm-derived pancreas. Indeed, some studies have reported generations of insulin-producing cells from ESC (Soria et al., 2000; Lumelsky et al., 2001). However, the insulin content was significantly lower in comparison with mature islet β-cells and when transplanted into animals, ESC-derived cells developed tumours (Fujikawa et al., 2005). In addition, the ESC research and eventual clinical application is hindered by serious ethical concerns.

Since the adult stem cells do not raise such ethical issues, they may be considered as a more suitable source of insulin-producing tissue. Moreover, adult stem cells are partially committed and are supposed to give rise to all cell types of the organ in which they reside. Thus, further manipulation of adult stem cells in an attempt to acquire fully differentiated cells with a mature phenotype would be much easier than in the case of uncommitted ESC.

A large body of evidence supports the assumption that in postnatal life newly formed β−cells are at least partially derived from the pancreatic stem cell. However, conclusive evidence of such a stem cell that can give rise to β−cells in the adult pancreas is still lacking. Pancreatic

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Abbreviations: DAPI – 4,6-diamidino-2-phenylindole; DM – diabetes mellitus; ESC – embryonic stem cell(s); HGF – hepatocyte growth factor; ILCC – islet-like cell cluster; ITS – insulin transferrin selenium; LIF – leukaemia inhibitory factor; MACS – magnetic activated cell sorting; NGN-3 – neurogenin-3; PI – propidium iodide; PSC – pancreatic stem cell(s); RT-PCR – reverse transcriptase polymerase chain reaction; SDF – stromal derived factor; TCSC – tissue-committed stem cell(s).

stem cells (PSC) are believed to reside within ductal tissue or the islets themselves. The notion that ductal tissue harbours PSC is supported by the mechanism of islet development during organogenesis when new islets arise from pancreatic ducts (Pictet et al., 1972; Edlund, 2002). Indeed, it has been reported in several studies that cultures of mouse and human ductal cells give rise to insulin-producing cells (Bonner-Weir et al., 2000; Ramiya et al. 2000; Gao et al., 2003). Similar results have been achieved in studies using islet-derived cells (Lechner et al., 2005). Although the transplantations of these cells were capable to treat diabetic animals in some of the cases, low insulin content and the possible contribution of β cells present in the original islet and ductal tissues may weaken the stem cell character of the applied cells. For instance, simple β-cell replication may explain the reappearance of insulin-producing cells (Dor et al., 2004).

The main reason why a putative pancreatic stem cell has not yet been discovered is the lack of specific markers that could be used to recognize and purify them. Cell surface markers in particular may be very valuable for the attempt to isolate and further study stem cells. One of the few cell surface molecules that are considered as a pancreatic stem cell marker is c-Met, a receptor for hepatocyte growth factor (HGF) (Suzuki et al., 2004). In addition to other cell surface markers, the c-Met is also expressed in the recently identified human tissue-committed stem cells (TCSC) (Kucia et al., 2004). Another surface molecule that belongs to the family of TCSC markers is CXCR4. The CXCR4 receptor and its ligand, stromal derived factor SDF-1α, are required for appropriate patterning during ontogeny and stimulate the growth and differentiation of critical cell types. Moreover, CXCR4/SDF-1α axis is an obligatory component in the maintenance of pancreatic duct cell survival, proliferation, and migration during pancreatic organogenesis and regeneration (Kayali et al., 2003). As noted above, the results of several studies suggest that pancreatic duct epithelium itself serves as a pool for progenitors and PSC that are able to migrate toward the existing or newly forming islets and differentiate into new β-cells. In this case, CXCR4 could play an important role in the process of stem cell differentiation and migration during islet neogenesis.

The aim of our study was to characterize the population of CXCR4-positive human pancreatic cells, their ability to differentiate toward insulin-producing cells and determine whether these cells share the markers of stem cells and pancreatic endocrine progenitors. Here we report that CXCR4-positive cells from the islet-depleted pancreatic fraction express markers of pancreatic endocrine progenitors (neurogenin-3, nestin) and markers of pluripotent stem cells (Oct-4, Nanog, ABCG2, CD133, CD117). Upon *in vitro* differentiation, these cells form islet-like cell clusters (ILCC) that produce key islet hormones and acquire a β-cell like phenotype.

Material and Methods

Tissue preparation

To isolate CXCR4-positive cells, we used human pancreatic acinar tissue that remains after the islet isolation and is discarded. 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´s Teaching Hospital.

Human islets and pancreatic tissue were isolated according to previously described methods (Shapiro et al., 2000). Briefly, islets were isolated from pancreata obtained from 7 donors (mean age 49 ± 18 years, range 29–69). The pancreatic duct was perfused with a cold enzyme mixture containing Liberase HI (Roche, Indianapolis, IN). Tissue was then transferred to a modified Ricordi chamber (Shapiro et al., 2000) and separated by gentle mechanical agitation and enzymatic digestion at 37°C. Islets were purified with the use of continuous gradients of Ficoll-diatrizoic acid in an aphaeresis system (model 2991, Gambro, Přerov, Czech Republic). The continuous Ficoll gradient was layered before the separation step. The solution densities ranged from 1.064 to 1.092 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 pooled and further processed.

Cell separation by magnetic activated cell sorting (MACS)

Cellular material obtained from islet isolation (3×10^8 cells) was filtered through a 70-μm cell strainer to remove residual islets. Filtered tissue suspension was digested with Accutase solution (Sigma-Aldrich, Steinheim, Germany) for 10 min at 37°C. The single-cell suspension was washed in staining buffer (PBS, 0.5 %HSA, 2 mM EDTA, pH 7.2) and centrifuged at 300 g for 10 min. Cell pellet was resuspended in staining buffer with diluted (1 μg/10^6 cells) primary mouse anti-CXCR4 antibody (SantaCruz Biotechnology, Santa Cruz, CA) for 10 min at 4°C. After washing in staining buffer and centrifugation (300 g, 10 min.), the cell pellet was incubated with microbeads conjugated to a monoclonal antibody against primary CXCR4 antibody Rat Anti-Mouse IgG2a+b MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min. at 4°C. After the final washing, cells were resuspended in staining buffer and processed in a MidiMACS magnetic separation unit (Miltenyi Biotech) with attached LS column (Miltenyi Biotech). The column was rinsed with cold staining buffer to wash out unlabelled cells. CXCR4-positive cells trapped in the second LS column were eluted after the column had been removed from the magnet field of the MidiMACS separation unit. The purity of cells was determined by immunofluorescence staining using goat anti-CXCR4 antibody (SantaCruz Biotechnology).

Cell culture studies

CXCR4-positive islet-derived pancreatic cells were pre-cultured for the first three days in CMRL 1066 medium (Sigma-Aldrich) containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM Hepes, 10 µl/ml Glutamax-I (Gibco BRL, Paisley, UK) and 2 mmol/l streptozotocin. On the 3rd day after isolation, cells were washed with HBSS (Biocoll, Seattle, WA) and further cultured in serum-free DMEM/F12 expansion medium supplemented with 0.5 % human albumin, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM Hepes, 1 mM sodium pyruvate, 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium (ITS) (all Sigma-Aldrich) and 10 µl/ml Glutamax-I (Gibco). To promote cell proliferation, the medium was supplemented with 10 ng/ml of basic fibroblast growth factor (FGF2) and 20 ng/ml epithelial growth factor (EGF) (both Peprotech, Rocky Hill, NJ). To induce differentiation into pancreatic endocrine cells, the medium was supplemented with 100 pM exendin-4 , 10 nM nicotinamide (both Sigma-Aldrich), 2 nM activin-A, 10 pM HGF and 2 nM LIF (all Peprotech), and cultured for one week.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA (from approximately 10^6 cells and 10^5 cells in case of ILC) was isolated using Rneasy Plus Mini Kit (Qiagen, Hilden, Germany) and treated with DNAse using RNase-Free DNase Set (Qiagen). RNA was isolated from approximately $1x10^6$ cells and 10^5 cells in case of ILCC. Isolated RNA (2 µg) was reverse transcribed with 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 positive control. Gene-specific primer pairs, annealing temperatures, and product sizes are listed in Table 1. All of the primers span at least one of the introns and most of them are designed to span the intron/exon boundary. PCR products were separated and visualized on 2% agarose gel containing ethidium bromide.

Table 1. Sequences of specific primer pairs employed for RT-PCR

Gene name	Forward primer	Reverse primer	Annealing temperature	Product length
Insulin	ccatcaagcacatcactgtcc	ccatctctctcggtgcagg	61	414
PDX-1	cttgttctcctccggctcc	cttgtcctcctccttttttccac	61	202
Glucagon	gcgagatttcccagaagagg	agcaggtgatgttgtgaagatg	61	198
GLP-1 Receptor	tggcattggggtgaacttcc	caggcgctggagtctcagc	61	462
Neurogenin-3	tctattcttttgcgccggtag	agtgccaactcgctcttagg	61	256
NeuroD/BETA2	aggaattcgcccacgcag	gtctcttgggcttttgatcgtc	61	346
Isl1-1	ggagcaactggtagagatgacg	agtactttccaaggtggctgg	61	245
Nkx2.2	ctacgacagcagcgacaacc	gccttggagaaaagcactcg	61	219
Nkx6.1	gctctacttcagccccagc	ggaaccagaccttgacctgac	61	332
OCT-4iso1	agctggagaaggagaagctgg	tcggaccacatccttctcgag	61	458
OCT-4iso2	ggggagattgataactggtgtg	tcggaccacatccttctcgag	61	480
ABCG2	gatatggatttacggctttgc	aaggccacgtgattcttcc	61	175
Nanog	gcaaacaacccacttctgc	aggccttctgcgtcacac	61	288
Glut2	cactgctgtctctgtattccttgtg	aaactcagccaccatgaacc	61	223
Pax4	actcccagtgtctcctccatc	ggaaaaccagaccctcacc	61	298
Pax6	gccctggagaaagagtttgag	gcgctgtaggtgtttgtgagg	61	314
HNF3beta	tggagcagctactatgcagagc	atggagttcatgttggcgtag	61	356
HNF6	cgcaggtcagcaatggaag	gatgagttgcctgaattggag	61	535
HLXB-9	taagatgcccgacttcaactcc	caaatcttcacctgggtctcg	61	192
LIFR	cttgcgagcctatacagatgg	ctgcttccctcacagacactc	61	249
HGF-R	agtgaagtggatggctttgg	gggcagtattcgggttgtag	61	162
CD117	agatgctcaagccgagtgc	actatcgctgcaggaagactcc	61	284
CD133	tcttcctcatggttggagttgg	ttaccagtctgagccaagtagc	61	478
GAPDH	ggagtcaacggatttggtcg	catgggtggaatcatattggaac	61	142
SOX17	cagaatccagacctgcacaac	ctgctcggggaactggag	61	294

Immunocytochemistry

MACS-separated cell suspension was washed with PBS and then centrifuged onto microscope slides at 55 g for 10 min. Cytospin slides were fixed in 4% PFA for 15 min and rinsed in PBS before staining.

ILCL were washed with PBS, fixed for 15 min in 4% PFA, rinsed with PBS, suspended in a 2% agarose-PBS solution and centrifuged at 100 g for 3 min to form compact pellets. After overnight submersion in 30% sucrose, ILCC were embedded in OCT mounting medium (TissueTek, Bayer Corp, Pittsburgh, PA), frozen in liquid nitrogen, and stored at –80°C.

After several washes in PBS, 8-μm sections of frozen ILCC or fixed cytospin slides were incubated in blocking solution containing 10% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA) 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 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 following dilutions: goat anti-CXCR4, 1 : 100 (SantaCruz Biotechnology), mouse anti-CXCR4, 1 : 100 (SantaCruz Biotechnology), goat anti-nestin, 1 : 100 (SantaCruz Biotechnology), rabbit anti-LIFR, 1 : 100 (SantaCruz Biotechnology), goat anti-glucagon, 1 : 100 (SantaCruz Biotechnology), rabbit anti-insulin, 1 : 100 (SantaCruz Biotechnology), mouse anti-human C-peptide 1 : 200 (ExBio, Czech Republic). 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 488 donkey anti-goat IgG Alexa fluor 546, Alexa Fluor 555 donkey anti-mouse IgG, Alexa Fluor 555 donkey anti-rabbit IgG (all from Molecular Probe, Eugene. OR). 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 (Model BX 41, Olympus, Japan).

C-peptide cell content

Cell protein extract was isolated using AllPrep RNA/Protein Kit (Qiagen). Human C-peptide was determined using a C-peptide IRMA kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions.

Results

Pancretic tissue obtained after islet isolation from human pancreata (N = 7) was filtered through a strainer to remove any remaining islets. Dithisone staining of tissue samples confirmed the absence of pancreatic islets, which typically stain red due to their zinc content. Cellular material was further dissociated to obtain single-cell suspension. The ratio of β-cells present within the cell suspension was assessed by immunofluorescence detection of C-peptide-positive cells and was as low as 0.04 ± SD 0.02 % (Fig. 1D).

CXCR4-positive cells represented 22.1 ± 6.6 % of all cells processed by MACS. The purity of cell fractions revealed by immunostaining was more than 90% in the case of CXCR4-positive as well as -negative fractions (Fig. 1A). Slight contamination of both fractions by β-cells was confirmed by RT-PCR (Fig. 2). The expression of transcription factors involved in pancreatic endocrine cell differentiation (Pdx1, NeuroD1, Pax6, Nkx2.2, Nkx6.1, Isl1 and HNF-3β) was also detected in both fractions (Fig. 2). Detection of these transcription factors could be caused by the presence of mature β-cells; however, other cell types could also express some of these transcription factors. In particular, the detection of the neurogenin-3 mRNA within CXCR4-positive cell population (Fig. 2) reveals the presence of pancreratic endocrine progenitors. Neurogenin-3 was also expressed in the islet cell fraction (Fig. 2).

Immunostaining for nestin, a putative marker of PSC, showed some positive cells in the CXCR4 cell fraction (Fig. 1B). Positive staining for leukaemia inhibitory factor receptor (LIFR) (Fig. 1C), another cell surface marker of TCSC, is in accordance with RT-PCR detection (Fig. 2). RT-PCR analysis further revealed the expression of several markers of pluripotent and adult stem cells, including transcription factors Oct4 and Nanog, and cell surface receptors CD117, CD133, and ABCG2.

To explore the potential of CXCR4-positive cells to differentiate toward islet endocrine cells we have decided to deplete contaminating β-cells. The objective of this step was to exclude the possible contribution of β−cells that could proliferate or re-differentiate during further culturing. After three days of cultivation in a medium containing streptozotocin and a subsequent 10 days of cultivation in a serum-free medium no insulin-positive cells were detected either by immunofluorescence or by RT-PCR.

The initial 3-day cultivation was accompanied with extensive cell apoptosis and necrosis that resulted in a 62.4 ± 8.2% reduction in the total cell number. During the 10-day culture in a serum-free medium, βFGF and EGF growth factors, which are known for their beneficial effects on proliferation of precursors, were added. The rate of cell apoptosis determined by PI staining had decreased to 8.7 ± 2.1% (data not shown). Concomitantly, there was an increase in the number of cells that reached 92.1 ± 15.1% of the original cell number. During cultivation, most of the cells were attached to the surface of the flask and possessed an epithelial-like, round morphology (Fig. 3A, B). The cell population obtained after cultivation in the serum-free medium no longer expressed insulin, glucagon, and somatostatin. On the other hand, the expression of

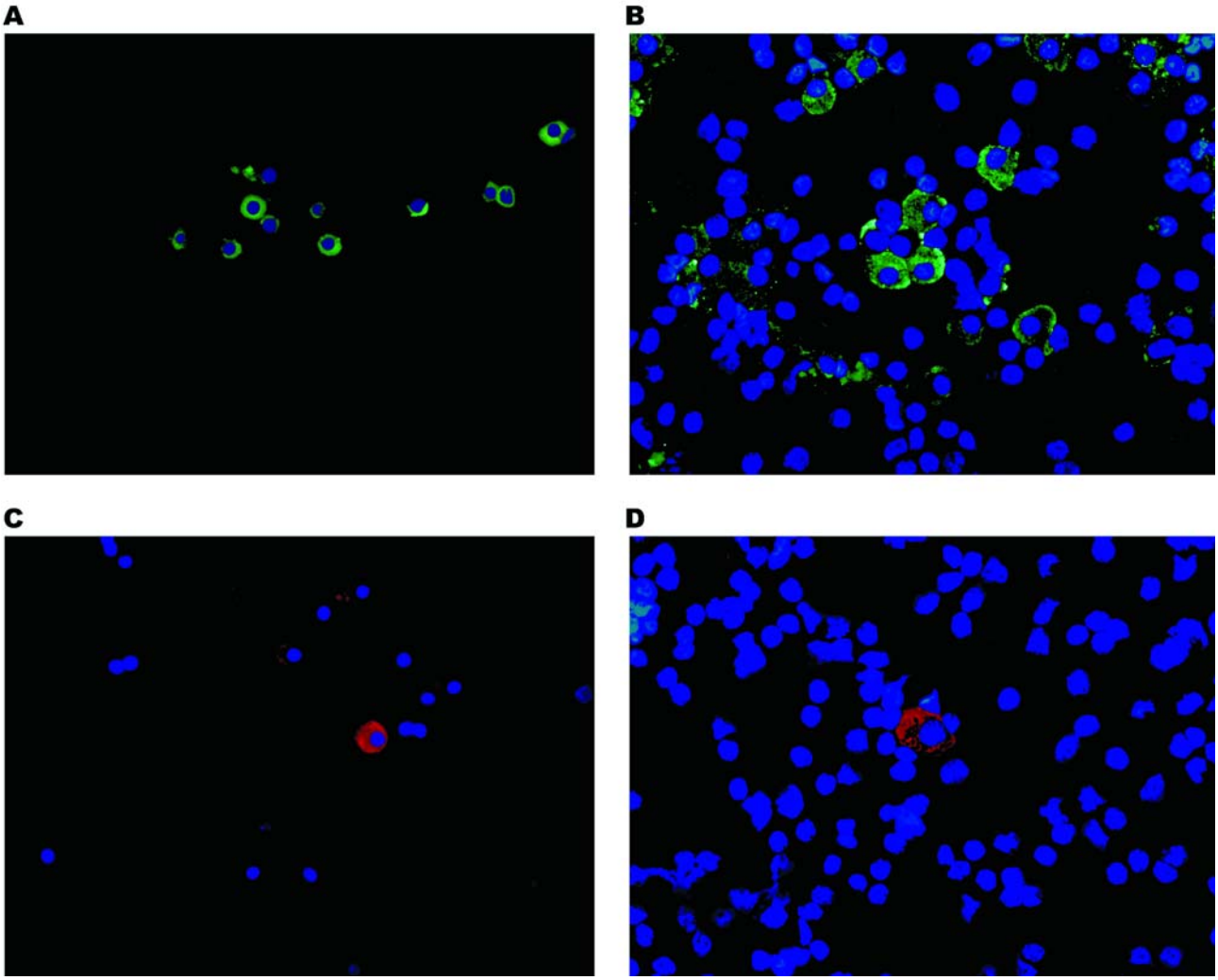

Fig. 1. Immunofluorescence detection of CXCR4 (A), nestin (B), LIFR (C) and insulin (D) in CXCR4 positive cells. All nuclei in A-D are stained blue with DAPI.

Pdx1, Nkx6.1, NeuroD1, Pax6, Isl1 and ngn-3 was still observed (Fig. 4).

Afterwards, cells were seeded at a high density in a medium supplemented with exendin-4, nicotinamide, activin-A, HGF and LIF, supplements that have been reported to support differentiation of insulin-producing cells. Two days after the initial seeding, some of the cells began to form colonies (Fig. 3C) that gave rise to the ILCC (Fig. 3D-F). The first ILCC were observed after four days of culturing in a differentiation medium (Fig. 3D). Within seven days, 108 ± 28 ILCC had formed from 5×10^6 cells. The average size of ILCC was 134 ± 87 μm and one of the ILCC contained 1231 ± 574 cells.

Differentiation of cells was associated with the expression of key pancreatic hormones insulin glucagon and somatostatin as well as trancription factors of endocrine differentiation (Fig. 4). The expression of preproinsulin and glucagon by ILCC was confirmed by immunostaining for both of these hormones (Fig. 5A). The proportion of ILCC C-peptide-positive cells (5.8 ± 1.1 %) was significantly lower than the number of glucagon-positive cells (13.3 ± 2.8) (Fig. 5B). C-peptide protein content was 0.38 ± 0.11 pmol/μg of total protein as determined by radioimmunoassay of ILCC cellular extracts. One of the most intriguing findings was almost homogenous staining for nestin in most of the ILCC cells (Fig. 5A). Within ILCC cells, CXCR4-positive cells were also detected (Fig. 5B).

Discussion

Here we report that the population of CXCR4-positive pancreatic cells contains endocrine progenitors that are able to give rise to different endocrine cells bearing characteristics of differentiated islet cells (β-cells, α-cells, δ-cells). Upon differentiation, CXCR4-positive cells isolated from non-endocrine pancreatic tissue form islet-like cell clusters that produce islet hormones such as insulin, glucagon, and somatostatin. In addition, we have shown that cells expressing markers of pluripotent and tissue-committed stem cells are present

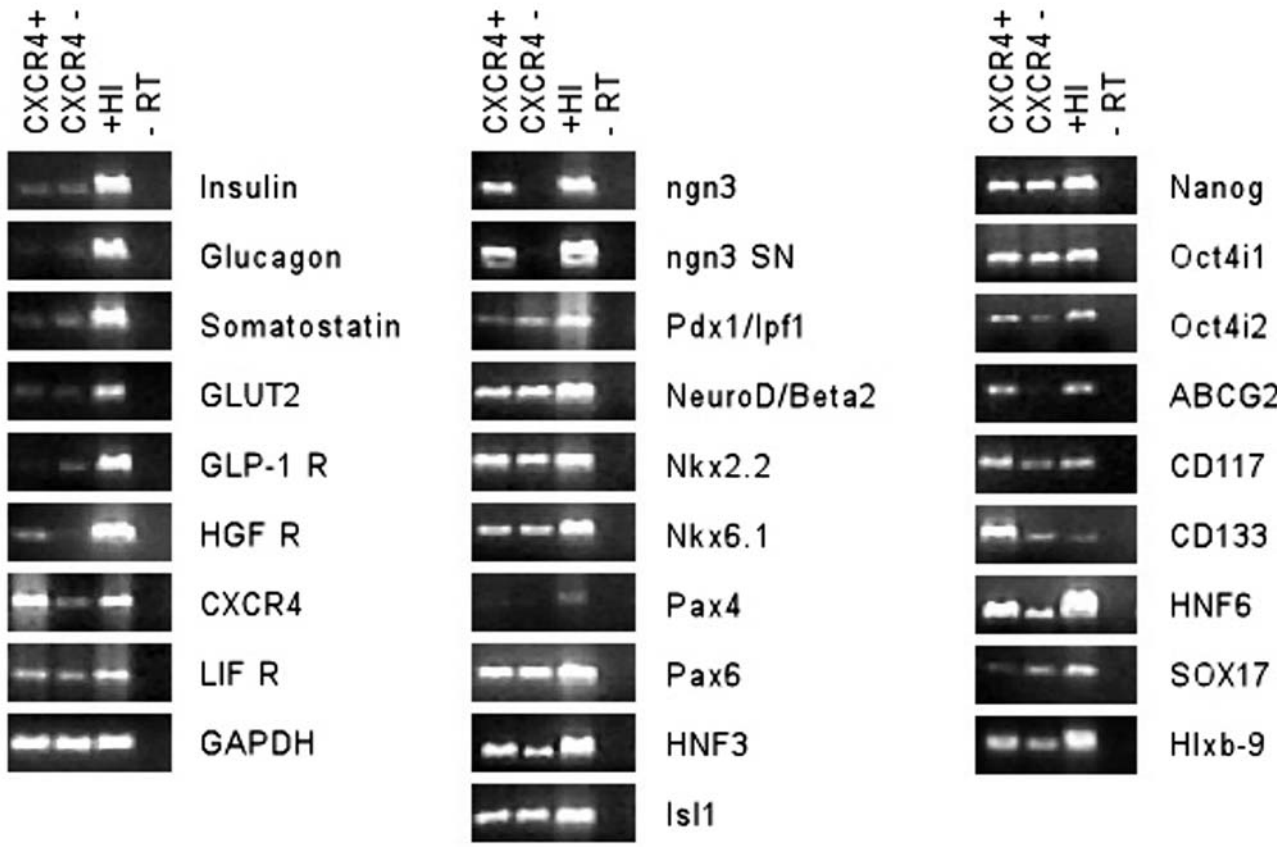

Fig. 2. RT-PCR analysis of gene expression in CXCR4-positive (CXCR4+) and CXCR-negative (CXCR4-) cells. Transcripts were analysed with the indicated primers, in comparison with a negative control (-RT) and a positive control (+HI, human islet RNA).

within the pancreatic non-endocrine tissue. These cells may represent the source of newly formed endocrine progenitors as well as differentiated islet endocrine cells.

In an attempt to identify a pancreatic endocrine progenitor and a stem cell, we have employed genetic markers that are involved in the development of endocrine pancreas and β-cell neogenesis. Among the best defined and studied markers are transcription factors that regulate gene expression during the differentiation process. Unfortunately, most of the transcription factors involved in β-cell differentiation could not be used as exclusive markers of endocrine progenitors since they are also expressed by mature endocrine cells and other pancreatic cell types (Edlund, 2002; Murtaugh et al, 2003). So far, the basic helix-loop-helix transcription factor neurogenin-3 (ngn-3) represents the only exception of this unfavourable rule.

The expression of ngn-3 is only transient. ngn-3 is exclusively expressed in islet cell progenitors and not in differentiated islet cells (Schwitzgebel et al., 2000). In rodents, ngn-3-positive cells give rise to all four endocrine cell types not only during embryogenesis, but also in postnatal life (Gu et al., 2002). In adult human pancreas ngn-3 expression was recently revealed only in islets (Lechner et al., 2005). It has led to the assumption that progenitor/stem cells exist only within islets, an idea supported by a few animal studies (Gu et al., 2002; Kodama et al., 2005). On the other hand, our finding that ngn-3-positive cells are present within adult non-endocrine tissue as well as in islets supports the hypothesis that newly formed β-cells bud from pancreatic ducts. Our result is in accordance with a study that employed a highly sensitive immunostaining method (Wang et al., 2005). In this study, ngn-3-positive cells were not only detected in islets of adult pancreas, but were also observed in duct-like cells of diabetes-prone rats. The discrepancy between these results and those obtained in earlier studies may stem from different immunostaining techniques and primers used for the detection of ngn-3 and its expression.

The expression of ngn-3 was more abundant in the CXCR4-positive cells in comparison with the CXCR4-negative population. Thus, it may be assumed that the CXCR4 receptor serves as a marker of pancreatic endocrine progenitors.

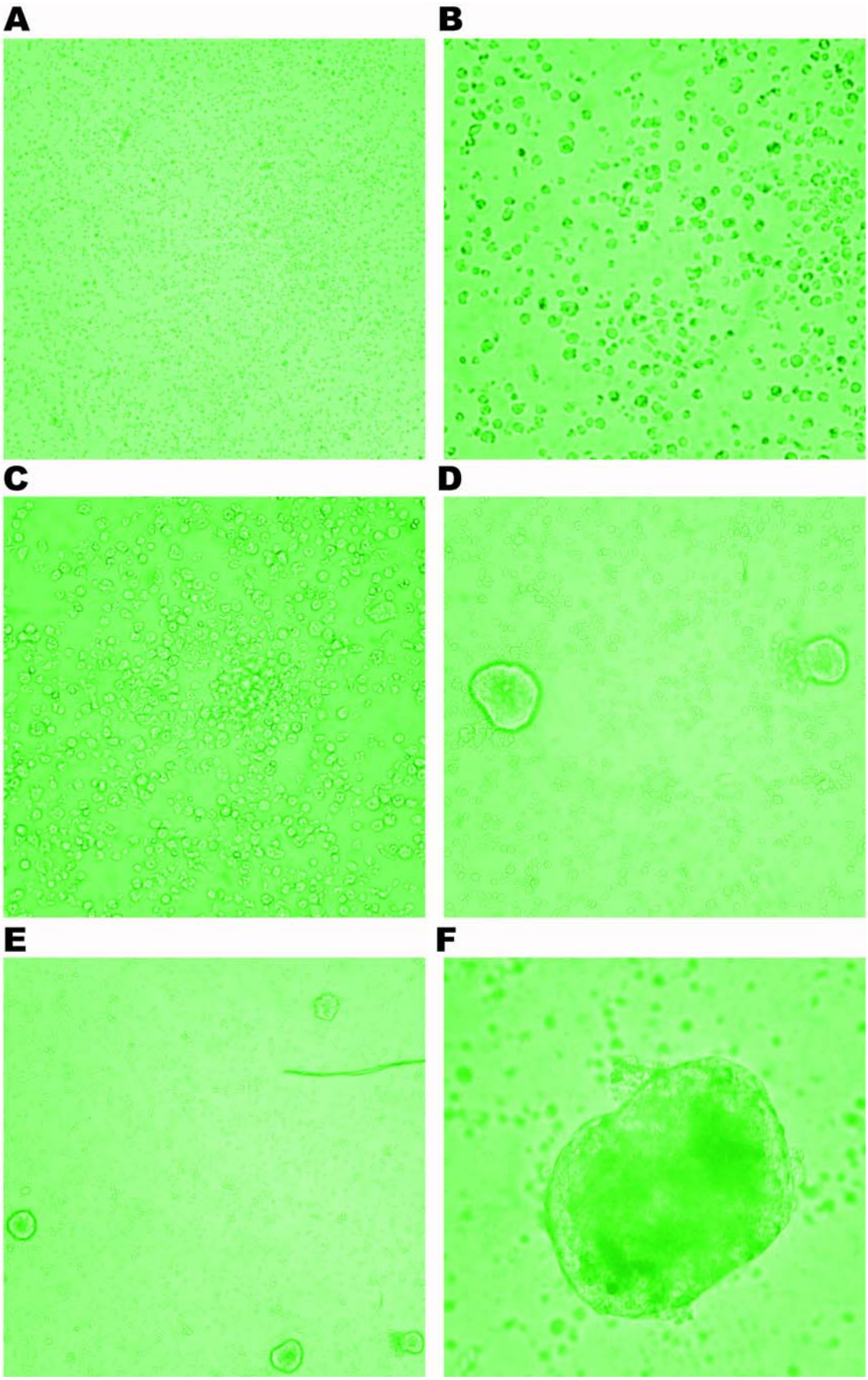

Fig. 3. Phase contrast images of: (A) cell suspension derived from CXCR4-positive cells, (B) 3x magnification of A, (C) forming cell clusters, (D-E) ILCC buds appear 4 days after the initiation of differentiation, (F) some of the ILCC attain a diameter of more than 200 μm.

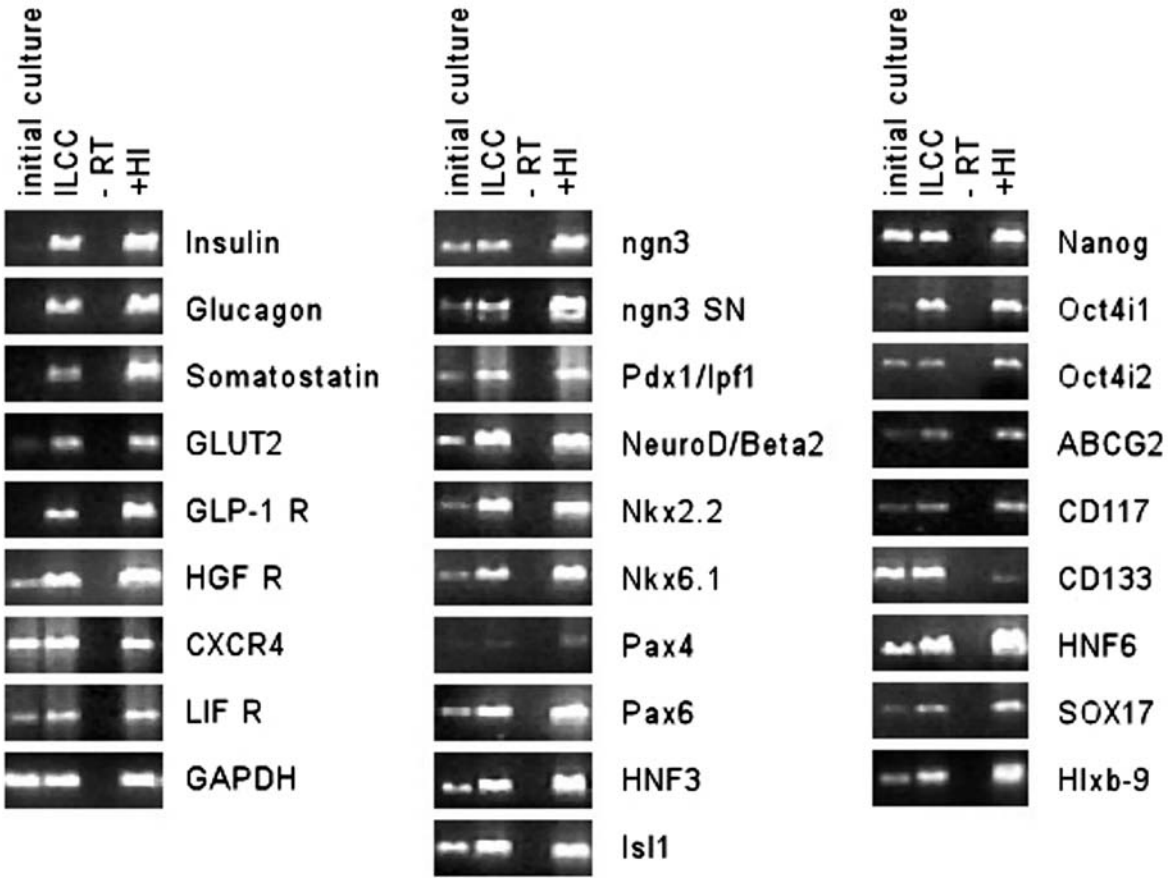

Fig. 4. RT-PCR analysis of gene expression in ILCC and initial culture prior to differentiation into ILCC. Transcripts were analysed with the indicated primers, in comparison with a negative control (-RT) and a positive control (+HI, human islet RNA).

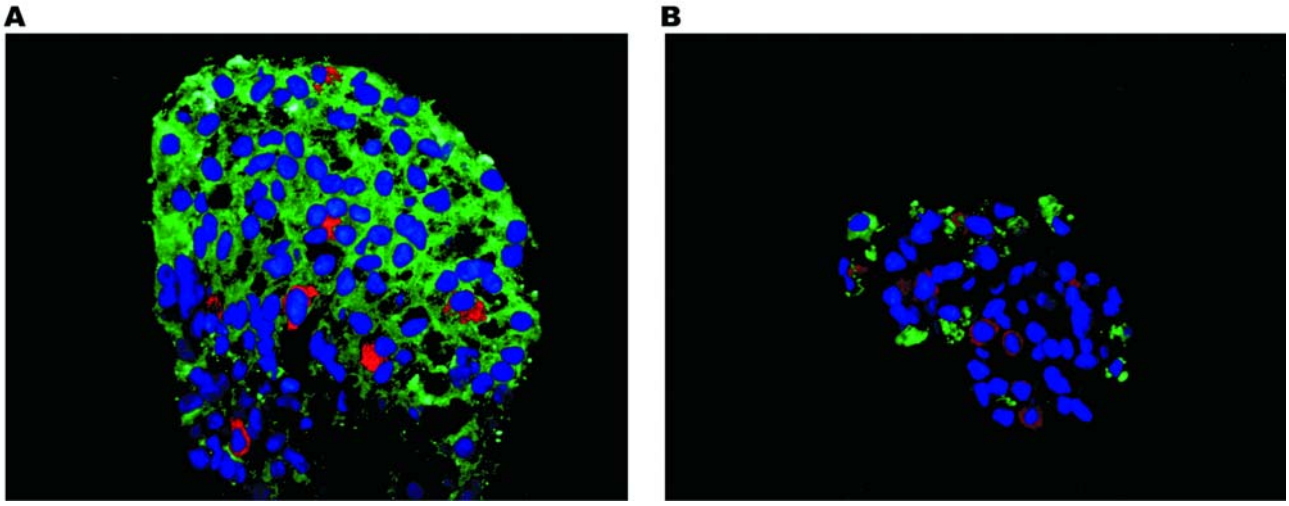

Fig. 5. Immunofluorescence double staining of C-peptide (red) and nestin (green) in ILCC (A), glucagon (green) and CXCR4 (red) in ILCC (B). All nuclei in panels A and B are stained blue with DAPI.

Based on this finding we wanted to determine whether the CXCR4-positive cell population also contains more primitive cells with stem cell characteristics. We have focused on the markers that are expressed by the pluripotent stem cells and TCSC. RT-PCR analysis revealed the expression of several markers of pluripotent stem cells including transcription factors Oct4 and Nanog that are required to maintain the pluripotency of embryonic cells (Cauffman et al., 2006), and markers of adult stem cells (Zhou et al., 2001; Yu et al., 2002;

Wang et al., 2004) such as receptor for stem cell factor CD117, cell surface receptor CD133, and the side population stem cell marker ABCG2.

The potential of CRCX4-positive cells that give rise to insulin-producing cells was tested by *in vitro* culture stimulated by selected growth factors and nutrients. Although the total number of insulin-positive cells within newly formed ILCC was significantly lower in comparison with native islets, the number of glucagon-positive cells was almost the same as in freshly isolated islets. It could have been caused by the culture medium used during differentiation. Our selection of growth factors and supplements could prefer α-cell differentiation of endocrine progenitors at the expense of β-cell differentiation.

Another interesting finding is that almost all the cells in ILCC were positive for nestin. Nestin is thought to be a marker of PSC that is expressed during differentiation of insulin-producing cells. Although the role of nestin as a marker of PSC has been disputed (Selander et al., 2002; Treutelaar et al., 2003), most of the studies addressing the expression of nestin during β-cell differentiation supports the conclusion that nestin marks PCS (Wang et al., 2005; Eberhardt et al. 2006). With a low proportion of hormone-positive cells within ILCC and the high number of nestin-positive cells we may assume that some hormone-negative progenitors or stem cells were still present within ILCC. This assumption is supported by the results of the RT-PCR that demonstrated the expression of ngn-3 and stem cell markers.

Although the formation of ILCC from pancreatic tissue has already been presented by other authors (Bonner-Weir et al., 2000; Ramiya et al. 2000; Gao et al., 2003; Wang et al., 2004; Lechner et al., 2005), our group is the first to describe the derivation of ILCC from CXCR4-positive pancreatic cells.

However, the mechanism of the differentiation of our ILCC could be explained by different means. In addition to the differentiation from stem cells, new β-cells could be derived by trans-differentiation from other pancreatic and even non-pancreatic cell types, which could have happened in our case as we used mainly acinar and ductal tissue. Another possible explanation could be de-differentiation of mature β-cells into a more primitive cell type such as progenitor or stem cells and their further re-differentiation into insulin-producing cells. However, the low β-cell number in the original sample does not necessarily support this explanation.

The formation of ILCC from the different cell types was described by many authors (Bonner-Weir et al., 2000; Ramiya et al. 2000; Gao et al., 2003; Lechner et al., 2005); however, only some of these experiments were successfully reproduced. In some of the cases, the detected insulin could be absorbed by cells from the culture medium which contained insulin supplements (Rajagopal, 2003; Hansson et al., 2004). In our study, for insulin production we employed the detection of C-peptide that is not added to the medium. C-peptide is a part of the pro-insulin peptide that is produced by β-cells and later separated into mature insulin and C-peptide.

The origin of newly formed β-cells in postnatal life still remains unclear. Although a large body of evidence supports the assumption that newly formed β cells are derived from PSC, the conclusive evidence of such specific stem cell type in the adult pancreas has not yet been presented. In our study, we have shown one possible source of β cells that could be easily obtained and, upon *in vitro* differentiation, give rise to insulin-producing cells. In future research, we will focus on the potential of these cells to produce insulin *in vivo* and possibly treat diabetic animals.

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