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Expression of Progenitor Cell Markers During Expansion of Sorted Human Pancreatic Beta Cells

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Functional pancreatic beta cell mass is dynamic and although fully differentiated, beta cells are capable of reentering the cell cycle upon appropriate stimuli. Stimulating regeneration-competent cells in situ is clearly the most desirable way to restore damaged tissue. Regeneration by dedifferentiation and transdifferentiation is a potential source of cells exhibiting a more developmentally immature phenotype and a wide differentiation potential. In this context and to gain a better understanding of the transformation induced in human beta cells during forced in vitro expansion, we focused on identifying differences in gene expression along with phenotypical transformation between proliferating and quiescent human beta cells. FACS-purified beta cells from three different human pancreata were cultured during 3–4 months (8–10 subcultures) on HTB-9 cell matrix with hepatocyte growth factor. Gene expression profiling was performed on cells from each subculture on "in-house" pancreas-specific microarrays consisting of 218 genes and concomitant morphological transformations were studied by immunocytochemistry. Immunocytochemical studies indicated a shift from epithelial to neuroepithelial cell phenotype, including progenitor cell features such as protein gene product 9.5 (PGP 9.5), Reg, vimentin, and neurogenin 3 protein expression. The expression of 49 genes was downregulated, including several markers of endocrine differentiation while 76 were induced by cell expansion including several markers of progenitor cells. Their pattern also argues for the transdifferentiation of beta cells into progenitor cells, demonstrating neuroepithelial features and overexpressing both PBX1, a homeodomain protein that can bind as a heterodimer with PDX1 and could switch the nature of its transcriptional activity, and neurogenin 3, a key factor for the generation of endocrine islet cells. Our study of the machinery that regulates human beta cell expansion and dedifferentiation may help elucidate some of the critical genes that control the formation of adult pancreatic progenitor cells and hence design targets to modify their expression in view of the production of insulin-secreting cells.

Key words: DNA microarray; Beta cell expansion; Gene expression; Human purified beta cell

RECENT advances in islet transplantation (42) highlight the urgent need to develop alternatives to human pancreas donors. Two main approaches are expected to achieve beta cell expansion: expansion of mature beta cells and differentiation of progenitor cells.

In mammal tissues such as muscle, bone, epithelia, blood, central nervous system, and pancreas, stem cells or progenitors cells can be activated during regeneration. In the pancreas, the regeneration process is triggered when insulin demands increase under physiological and pathological conditions. In vertebrates with more extensive regenerative abilities such as in salamanders, new stem cells or progenitor cells are created through a process of cellular dedifferenti-

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ation in which differentiated cells can reverse the normal developmental processes and once again became precursor cells (28). The reversibility of a differentiation program, termed dedifferentiation or retrodifferentiation, opens a wide range of new possibilities for cellular development. During differentiation and retrodifferentiation, the expression of gene products associated with a differentiated phenotype and cell cycle regulation demonstrates opposite patterns. This effect requires a coordinated network that simultaneously controls cell growth and differentiation (17). Human beta cells can be stimulated to divide in vitro, on a complex extracellular matrix in the presence of hepatocyte growth factor, but this expansion features a loss of differentiation patterns; in particular, insulin is no longer synthesized (3,4). By analyzing cDNA "in-house" arrays and cell immunostaining, we examined the genes implicated in cell cycle regulation and cellular dedifferentiation in expanding human beta cells as well as their phenotypical transformation. We postulated that these expanded cells might represent progenitors that can be primed to preferably redifferentiate into insulin-secreting cells, provided they could find an appropriate environment.

This study was thus designed to identify the genes implicated in beta cell replication and beta cell function that are likely to be mutually exclusive. Our aim in so doing was to emphasize the signals that can induce the differentiation of stem/progenitor cells into functional cells and the signals that maintain viability and a correct physiologic state in the differentiated cells.

MATERIALS AND METHODS

Human Islet Processing

Human pancreata (mean donor age: 44 ± 13 years, $n = 3$) were harvested from adult brain-dead donors in agreement with French Regulations and with the local Institutional Ethical Committee. Pancreatic islets were isolated after ductal distension of the pancreas and digestion of the tissue with Liberase® (Roche Diagnostics, Meylan, France) according to a slightly modified version (21) of the automated method of Ricordi et al. (37). Semipurification was achieved with Euro-Ficoll continuous density gradients using a COBE 2991 cell separator (23). The number of islets was determined on samples of each preparation after dithizone staining and expressed as an equivalent number of islets (IE) (36). Preparations used in this study exhibited a 82 ± 7% purity and an average yield of 3635 ± 642 IE/g pancreas. Semipurified islets were cultured in CMRL 1066 medium (Gibco BRL, Life Technologies, Cergy-Pontoise, France) containing 10% fetal calf serum (FCS, Euro Bio Laboratories, Les Ulis, France), penicillin (100 μUI/ml) and streptomycin (100 μg/ml).

Beta Cell Separation

Single purified beta cells were obtained by gentle pipetting of islet cells for 4–6 min in the enzymatic dissociation buffer Splitase from Autogen Bioclar UK Ltd (Calne, Witts, UK). The reaction was stopped with FCS, 3:1 v/v when about 80% of islet cells appeared as single cells. Cell suspension was centrifuged and the pellet was suspended in F10 medium containing 2.8 mM glucose. After a recovery period of 90 min, cells were stained with the zinc-sensitive fluorescent probe Newport Green diacetate (Molecular Probes Europe, Leiden, The Netherlands) as previously described (27) and the cell suspension was filtered through a 70-μm nylon screen. Sorting was achieved using a FACS Vantage flow cytometer equipped with an argon-ion laser (Becton Dickinson, Rungis, France). Excitation was performed with the 488-nm blue line of the laser and emission measured through a 530/30 bandpass filter.

Cell Culture

FACS-purified beta cells were cultured on human tumor bladder-9 matrix obtained by destruction of HTB-9 cells (ATCC, Rockville, MD) with a 0.28% ammonium hydroxide solution. Culture media was CMRL 1066 without phenol red and supplemented with 25 mM HEPES, 10 mM nicotinamide, 6 mM sodium pyruvate, and 10 ng/ml hepatocyte growth factor (HGF), all of which are reagents from Sigma Aldrich Chemicals. Every 3 days, media were replaced by fresh media. This was repeated for a period of 3–4 months.

Immunocytochemistry

Immunocytochemistry was performed on cell cytospins fixed in 1% paraformaldehyde. Table 1 details antibody specificity and dilution. Antibodies were revealed with EnVision system using 3,3-diaminobenzidine (DAB) (Dako, Trappes, France) or phtalocyanin red (Histomark Red, Kirkegaard and Perry Laboratories, Gaithersburg, MD). Controls included replacing the primary antibody with PBS containing bovine serum albumin (1%). Nuclei were counterstained with Carazzi's hematoxylin. Hoechst staining (Bisbenzidine 33258, 10 μg/ml) of cell nuclei was performed for 10 min at 37°C, and Newport Green staining (Newport Green potassium salt, 50 μg/ml, Molecular Probes) of insulin-containing cells was performed at 37°C for 30 min.

Expression Profiling of Expanded Human Beta Cells by cDNA Microarrays

Glass Microarray Preparation. The oligonucleotides used in this study were 70 mers defined using the OMAD data base (http://www.omad.qiagen.com/download/storage/human_V3.0.0_genelist_s-.xls.gz) provided by Operon (Qiagen S.A, Courtaboeuf, France). Each 70 mer probe was designed with a bias towards the 3′ end of the gene. The sequence of each probe was optimized using the search program Basic Local Alignment Search Tool (BLAST) by selecting the region of maximal specificity to the target gene while minimizing cross-hybridization to other genes.

Each oligonucleotide consisted in a lyophilized powder (200 nmol), which is sufficient for printing at least 1000 slides. Probes were printed on γ-aminopropyl silane-coated glass slides (Corning) with a VersArray ChipWriter Pro system arrayer (Bio-Rad, Marnes-la-Coquette, France). Each gene was spotted in triplicate (50 fmol/spot) from a 50-μM solution in 3× saline sodium citrate buffer (SSC, 0.3 M sodium citrate, 3 M NaCl, pH 7).

There were 218 elements on the pancreatic DNA microarray including growth and transcription factors, hormones, glucose transport and metabolism proteins, oxidative stress and signal transduction proteins.

Information about the pancreatic DNA microarray is available at http://www.univ-lille2.fr/ilots/microarray/micro.html

Preparation of RNA. Total RNA was extracted from proliferating purified human beta cell at each passage of the culture period. Expanded cells were lysed in a 1% β-mercaptoethanol-containing buffer obtained from an RNA extraction kit (Macherey Nagel, Hoerdt, France); RNA was extracted as described by the manufacturer. The quality of total RNA was assessed with the Agilent RNA 6000 chips coupled with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), by visualizing the 18S and 28S ribosomal ribonucleic acid (rRNA). All RNA used in this study met the minimum requirement of at least a 1.8 ratio of 18S:28S rRNA. The RNA aliquots were stored at −80°C before use.

RNA Labeling. The cDNAs were synthesized from 5 μg of DNA-free total RNA previously incubated for 10 min at 65°C with 1.5 μl hexanucleotide mix 10x (Roche Diagnostics). Reverse transcription (RT) was performed at 42°C for 1 h by adding 200 U reverse transcriptase (Superscript II, Invitrogen, Cergy-Pontoise, France) plus 2.5 mM of each dNTP (Amersham Pharmacia, Saint Quentin Yvelines, France). cDNAs were labeled by incorporation of 1.5 μl Cy-5-dUTP or Cy-3-dUTP (25 nM each, Amersham Pharmacia). At the end of the RT, 0.5 μl of reverse trancriptase was added for a new heating cycle of 45 min at 42°C. The final step was performed at 66°C for 30 min by adding 7.5 μl NaOH/EDTA (0.1 N/2 mM) that was finally inactivated with 7.5 μl HCl 0.1 N.

Labeled cDNAs were purified through a centrifugal filter (Microcon YM-30, Millipore Ltd, Watford, UK). cDNAs were diluted in a final volume of 500 μl Tris/EDTA (TE) buffer. Samples were centrifuged for 12 min at 11000 rpm and washed two times in 450 μl TE buffer. The final elution step consisted of centrifugation for 20 s at 3000 rpm. Samples were stored at −20°C before use.

Prehybridization. Microarrays were first incubated for 30 min at 42°C in the prehybridization solution (Chip Spread, Ventana Medical Systems Inc, Tucson, AZ), rinsed several times at room temperature, and then incubated for 10 min at 70°C in Chip Prep 1 solution and for 30 min at room temperature in Chip Prep 2. Samples were laid on slides for a denaturation step of 2 min at 37°C followed by a second step of 2 min at 45°C. Slides were then ready for hybridization.

Hybridization. Before hybridization on pancreatic DNA microarray, Cy-3- and Cy-5-labeled cDNA were combined with 200 μl hybridization solution (Ventana Medical Systems) and denatured for 5 min at 95°C.

Hybridization and cleaning were achieved according to an automated method using a Ventana Discovery™ system (Ventana Medical Systems). Slides were hybridized for 8 h at 45°C and then washed several times as described in the Ventana protocol, which includes posthybridization washes at room temperature in 2× SSC. At the end of the hybridization step, slides were first manually washed at room temperature in RiboWash™ (10× SSPE, saline sodium phosphate EDTA, buffer, Ventana Medical Systems) for 1 min, then for 1 min in deionized water, and finally in ethanol 95°C for 1 min. Slides were then dried by centrifugation in a 50 ml conical tube for 6 min at 3000 rpm in an Eppendorf Centrifuge 5810R.

Scanning and Image Analysis. Following hybridization and washings, slides were immediately scanned at a resolution of 20 μm per pixel using an Affymetrix 428 scanner (Affymetrix Ltd, High Wycombe, UK). The resulting 16-bit TIFF images were analyzed with the Jaguar® software, which is provided with the scanner.

Data Processing and Normalization. Comparative measurements of transcript abundance were performed. Time course samples were analyzed by directly comparing the abundance of each gene's transcript relative to the control (noncultured cells) sample of the same pancreas. RNA samples taken during the time course were labeled with Cy-5, and RNA from the reference (HepG2 cells, ATCC) was labeled with Cy-3. HepG2 is a cell line originated from a human hepatocarcinoma and was used for normalization between microarrays.

Quantitative Real-Time Gene Expression

The cDNAs were synthesized from 2 μg of DNA-free total RNA incubated with 2 μl hexanucleotide mix 10x (Roche Diagnostics) and 4 U RNAsin (Gibco BRL) for 10 min at 70°C. Reverse transcription was performed at 42°C for 1 h by adding 200 U M-MLV reverse transcriptase (Life Technologies) plus 2 mM of each dNTP (Pharmacia, Saint Quentin Yvelines, France), 4 U RNAsin, and 1 μg tRNA (Boehringer Mannheim, Meylan, France). Products were further incubated for 1 h at 42°C using 100 U reverse transcriptase. The final reaction mixture was heated at 95°C for 5 min and stored at −20°C until used.

The mRNA was monitored in 96-well plates by quantitative RT-PCR of the target fragments and 18S and β-actin housekeeping gene sequences as internal standards. Amplification was achieved with specific primers of the target sequence (Table 2).

The template concentration per reaction represented one tenth of the cDNA reaction performed on 2 μg total RNA. Amplification was achieved in 25 μl reaction mixture containing 3 μl cDNA, 25 pM of each oligonucleotide primer, and 2× Sybr Green 4309155 Master Mix (Applied Biosystems, Courtaboeuf, France).

After denaturation for 10 min at 95°C, solutions underwent 40 cycles of amplification in a ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Amplification parameters included 30-s denaturation at 94°C and a 1-min annealing step at 60°C. Direct detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of Sybr Green to double-stranded (ds) DNA.

All quantitations were achieved with the comparative CT method and normalized to the two endogenous control 18S and β-actin mRNAs as described by the manufacturer.

RESULTS

Three human pancreata were processed for this study. The mean viability of isolated pancreatic islets was 95.3 ± 2.2% (mean ± SE). Dissociated pancreatic islet cells were FACS purified. The resulting viable beta cells exhibited a purity of 98.60 ± 0.55% as determined by insulin immunostaining. Beta cells plated (50,000 cells/cm^2) in 25-cm^2 culture flasks coated with HTB-9 matrix were 10-fold subcultured to confluency and reseeded at 1:10. During culture, beta cells demonstrated no signs of apoptosis as determined by Hoechst staining (Fig. 1a). They rapidly became fibroblast-like and, as previously described (5), only 20% of starting cells were still immunoreactive for insulin at day 14 of culture; thereafter, from day 28 onwards, they completely lost insulin immunoreactivity (Fig. 1a). Cells displayed continuous growth during the 3–4 month culture period corresponding to a 10^9-fold expansion. Cells from each subculture were analyzed at the gene and protein expression level by DNA microarray and immunocytochemistry analyses.

Immunocytochemical Study

Cell phenotypical transformations are depicted in Figures 1a and b and the description of the antibodies used is detailed in Table 1. The epithelial nature of our initial cells was ascertained by the positive immunostaining of the whole cell population with the anti-human epithelial antigen Ber-EP4, which does not label mesenchymal tissue. Moreover, no staining with the myofibroblast cell marker α-smooth muscle actin antibody was noted. In contrast, cells were immunoreactive for the neuroendocrine cell markers chromogranin A and synaptophysin. During culture Ber-EP4-, chromogranin A-, and synaptophysin-stained cells disappeared and, from subculture 4 on, they no longer exhibited positive Ber-EP4 and chromatogranin A staining. In contrast, PGP 9.5, Reg, vimentin, and neurogenin 3 positive-stained cells appeared at that time, and a reexpression of synaptophysin-stained cells was noted. Only few cells (<2%) of the starting population were positive for the ductal cell marker CK-19 and the exocrine cell marker amylase and they rapidly disappeared during culture. No cells were immunoreactive for the endothelial CD 31 (Pecam-1) marker nor for the neuroepithelial marker nestin.

Gene Expression Analysis

Our "in-house" arrays contained 218 individual triple-spotted genes. The background comprised ≤1.2% of the total signal for each array whereas intensities for spot values covered ≅50-fold range from 1.5 to 80%.

To demonstrate the basic characteristics of our chips we first checked the reproducibility of intensity

values within and between arrays. The mean standard error within triplicate values was $5 \pm 1\%$. The correlations between assays summarized in Table 2 first demonstrated that a satisfactory correlation between Cy5 intensity values was observed regardless of hybridization timing (performed at the same time or not). Secondly, we found that Cy5 labeling of pancreatic cDNA was not spoiled by the simultaneous spotting of Cy3-labeled cDNA from HepG2 cells; and finally that labeling of the same total pancreas with both Cy3 and Cy5 followed by hybridization on the same array resulted in a "same versus same" hybridization.

To minimize variations between assays, we used 30 arrays that had been spotted at the same time and microarrays from the same pancreas were processed together. Spot Cy5 intensities from noncultured cells and subsequent subcultures from a same pancreas were normalized using the mean intensity of Cy3-labeled cDNA from a pool of HepG2 cells that had been spotted on the same slides. The normalized values were used to calculate the ratios of the different time points versus control (noncultured) cells. The experiments were carried out with cells from three different pancreata. The final value for a spot position was the median of the three ratios.

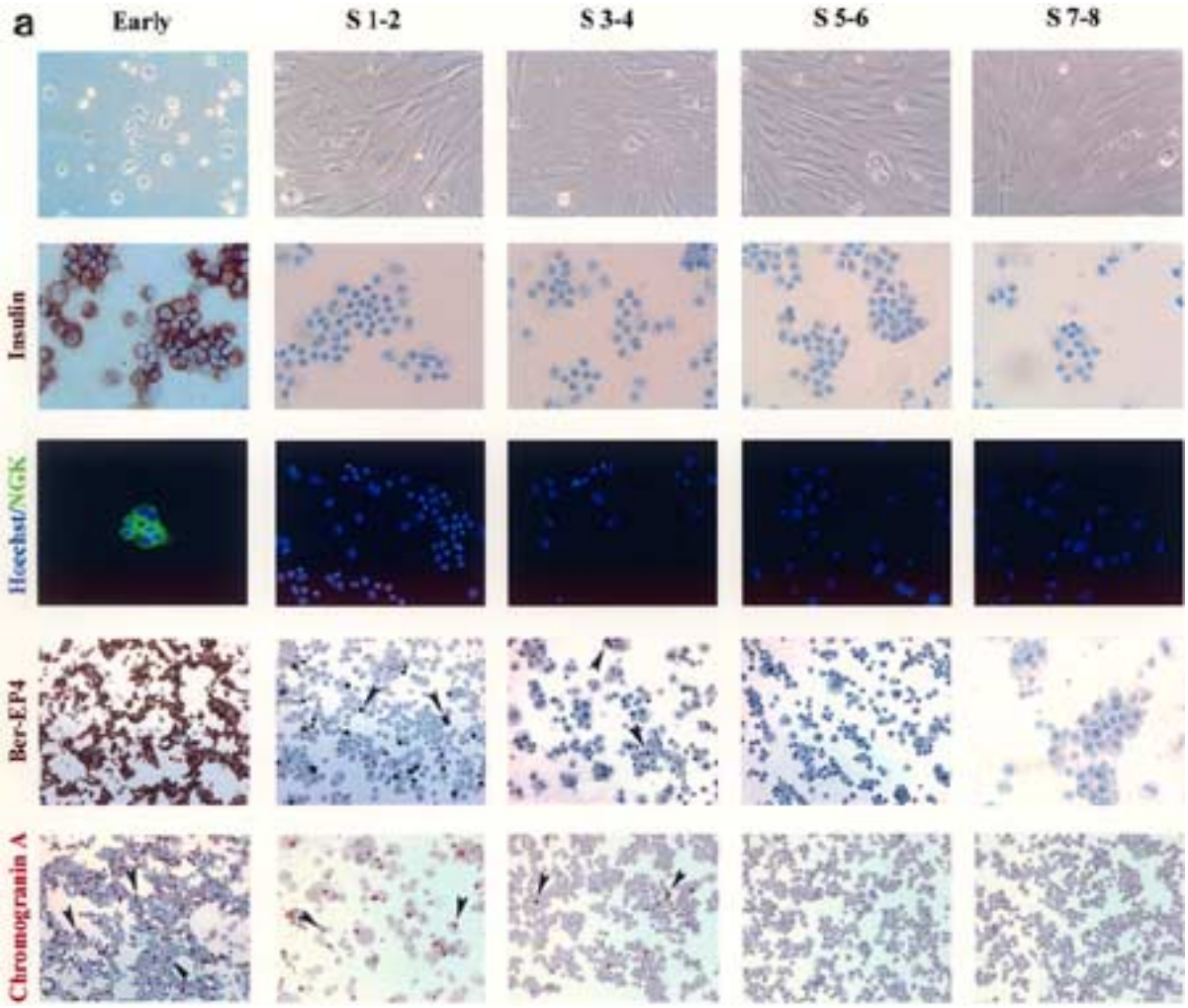
These data were then preprocessed using GEPAS (Gene Expression Pattern Analysis Suite v 1.0) to handle replicates and perform log-transformation, filtering, and normalization. The resulting datasets were then sent to the EPclust (@EBI). Among the 218 genes, 32 low-abundance transcripts were omitted, including among the most important NeuroD, Glut1, and Glut3. A positive expression of 186 transcripts was demonstrated including 61 genes exhibiting unchanged expression (i.e., fold-change less than 2), 49 genes that were downregulated, and 76 genes were induced by beta cell growth. Each of the two latter classes was separated into three clusters, one showing almost constant increase or decrease during the culture periods and the two others principally regulated at the beginning or at the end of the culture periods. Each value is the median of the three values obtained from three different pancreata. As shown in Figures 2–4, our data distribution is divided into seven clusters. Three clusters of genes were shut off by beta cell growth, particularly those characteristic of beta cell differentiation, such as insulin promoting factor-1 (IPF-1/PDX-1), insulin, uncoupling protein I and 3 (UCP1, UCP3), glucokinase, hexokinase, glucose 6 phosphate dehydrogenase, and insulin receptor substrate (IRS2), while three others were upregulated, particularly those described in regenerating or progenitor cells such as Reg1B, PGP 9.5, Glut 2, and neurogenin 3, or characteristic of neuroepithelial cells, whether primitive or not, such as vimentin, tyrosine hydroxylase, neuropeptide Y, neuron specific enolase, and neuronatin. To support our results we verified the expression of some of the genes by real-time RT-PCR, namely, insulin, IPF-1/PDX-1, chromogranin A, N-CAM, and RARβ for the group of downregulated genes, and PBX-1, Akt-1, neuronatin, neurogenin 3, and tyrosine hydroxylase for the group of induced genes. The primer sequences are listed in Table 3. The results shown in Figure 5 confirm the appropriate classification of these genes.

DISCUSSION

In a population of fully differentiated adult pancreatic islet cells, the number of beta cells actually undergoing cell division is small, with an estimated 0.5–2% (44). Upon appropriate stimulation, beta cells are capable of reentering the cell cycle. The entry of primary human beta cells into the cell cycle can notably be achieved when cells were cultured on extracellular matrix in the presence of HGF. However, this combination is limited by loss of differentiated function, particularly pancreatic hormone expression (4,16).

To highlight the genes implicated in beta cell dedifferentiation during the proliferation process, along with genes identifying transformed cells, we performed an "in-house" microarray kinetic study of FACS-purified human beta cells using Newport Green, a Zn-sensitive fluorescent probe that selects the insulin-positive cells, the mature as well as the possible small-size cells described by Petropavlovskaia and Rosenberg (34). During the same 3–4 month growth periods corresponding to 10 subcultures we also tried to phenotype the expanded cells.

Cells that were of epithelial origin, as demonstrated by their 100% immunostaining with the anti-human epithelial antigen Ber-EP4, were insulin positive at $98.5 \pm 0.6\%$. Insulin immunoreactivity gradually declined and was entirely lost at the first subculture corresponding to 30 days of culture. Meanwhile, cells grew more and more rapidly and could therefore be passaged every 15 days from day 30 ($\approx$S1) on. These cells remained immunoreactive for chromogranin A, a marker of neuroendocrine differentiation, until the fourth subculture and started to express vimentin, a marker of primitive neuroepithelial cells, neurogenin 3, a marker of endocrine progenitor cells, as well as markers of regeneration such as Reg and PGP 9.5. Cells also reexpressed synaptophysin, a marker of neuroendocrine differentiation. Throughout the entire culture, cells never expressed the myofibroblast cell marker α-smooth muscle actin, demonstrating that vi-



(*continued*)

Figure 1. Morphological and immunocytochemical characteristics (cytospins) of growing beta cells from early cultures (<14 days) to late cultures [subcultures 1 to 8 (S1–S8), from 1 to 3–4 months]. Antibody specificities are described in Materials and Methods. NGK: Newport Green probe (potassic form) that specifically stains insulin. Note that cells were also not immunoreactive for α-smooth muscle actin and nestin antibodies (images not shown). Arrowheads show a few of the positively stained cells.

mentin expression was not the result of myofibroblast contamination. In addition, cells were not immunoreactive for nestin, an intermediate filament protein described within the islet fraction and in neuroepithelial stem cells. These findings are in accordance with those of others (10,19,22,49), which demonstrate that nestin is not a specific marker of beta cell precursors in the developing pancreas. Our cells were also negative for the endothelial cell marker CD 31 (Pecam-1). The initial population consisted of 1–2% non-beta cells including ductal cells (CK-19-positive cells) and acinar cells (amylase-positive cells), which rapidly disappeared in our culture conditions, contrary to other reports that found an increase of CK-19-expressing cells along with an absence of growing beta cells in whole islet long-term culture (25). We suggest that the divergences between data could be explained by our starting material consisting in a nearly pure sorted beta cell population in which the very few scattered ductal or acinar cells had not proliferated, perhaps due to the lack of paracrine factors from neighboring cells or as reported by others (8,26,50), due to their low levels of HGF receptors, in contrast to developing beta cells. Moreover, these latter cells, which rapidly lose their differentiated properties, namely insulin secretion, could have been missed by insulin immunostaining.

Concerning gene expression, we first noticed as previously described (5), that PDX1/IPF1 was strongly downregulated (10-fold). This homeobox protein has a critical function in pancreatic development and glucose homeostasis. PDX1 stimulates the expression of

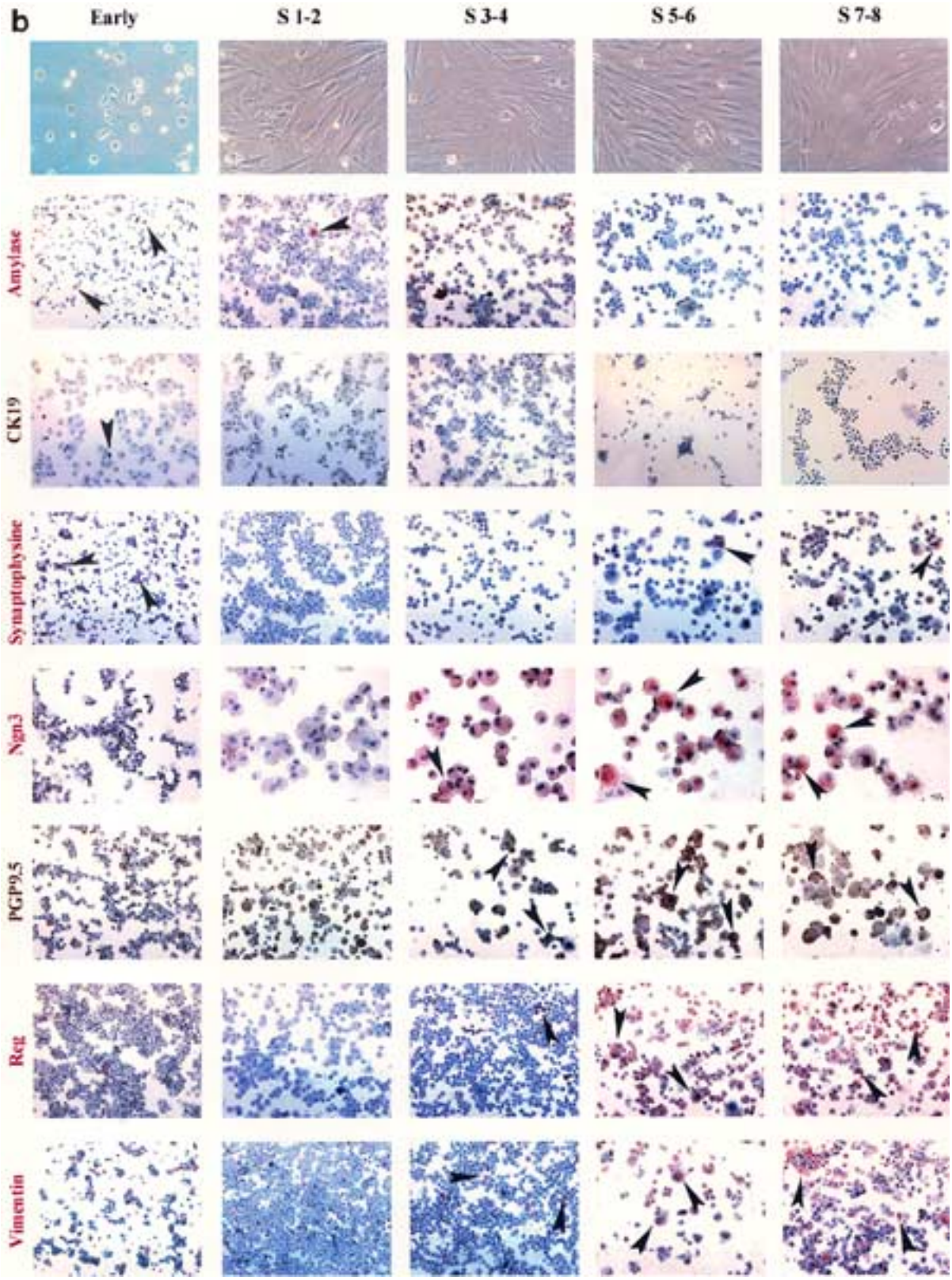


Figure 1. Continued.

TABLE 1
CHARACTERISTICS OF ANTIBODIES USED IN IMMUNOCYTOCHEMISTRY

Antibodies	Source	Type	Dilution	Specificity
Ber EP4	Dako	monoclonal	1:200	epithelial cells
Insulin	Biogenesis	monoclonal	1:500	beta cells
CK-19	Dako	monoclonal	1:100	ductular cells
Amylase	Sigma	polyclonal	1:400	acinar cells
Chromogranin A	Dako	polyclonal	1:400	neuroendocrine cells
Synaptophysin	Dako	polyclonal	1:300	neuroendocrine cells
Vimentin	Dako	monoclonal	1:400	neuroepithelial precursor cells
Ngn3	Transduction Laboratories	monoclonal	1:100	endocrine precursor cells
PGP 9.5	Novo Castra	monoclonal	1:200	neuronal cells
Reg	Dr. Figarella*	polyclonal	1:200	regenerating cells
CD 31/PECAM	Dako	monoclonal	1:100	endothelial cells
Nestin	Chemicon	monoclonal	1:100	intermediate filament
α-Smooth muscle actin	Biogenex	monoclonal	1:300	myofibroblasts

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certain islet-specific genes such as somatostatin, insulin, and glucokinase by binding as a monomer to promoter sites, genes that were also downregulated in our culture conditions. PDX1 also binds as a heterodimer with the ubiquitous homeodomain protein PBX1; this binding is thought to switch the nature of the transcriptional activity of PDX1. During culture, we noticed a strong induction of PBX1 that could potentially have combined with residual PDX1. PDX1/PBX complexes have been observed in ductal and acinar cell lines where they regulate the expression of exocrine target genes such as elastase (45). These complexes have also been shown to be likely most abundant in ducts of the developing pancreas and to chiefly induce the expression of genes involved in islet neogenesis (12), in contrast to beta cell lines where the complexes did not form (45). The important increase in PBX1 expression we noticed during cell expansion could be related, as described immunohistochemically by Schmied et al. (39), to overexpression, in our transformed cells, of genes typical of exocrine features (carbonic anhydrase II, elastase, α1-antitrypsin, Muc 5), which could not come from the scarce contaminating cells in our initial population because transformed cells did not express the ductal and acinar markers CK-19 and amylase. In contrast, transformed cells overexpressed several markers of regeneration and progenitor cells.

TABLE 2
REPRODUCIBILITY STUDY OF THE INTENSITY VALUES ACCORDING TO MICROARRAY EXPERIMENT CONDITIONS

Pancreas	Hybridization	Array	Correlation Coefficient
Pancreas (Cy5/Cy5)	same time	different	$r^2 = 0.923$
Pancreas (Cy5/Cy5)	different time	different	$r^2 = 0.904$
Pancreas + HepG2 (Cy3 + Cy5/Cy5)	same time	different	$r^2 = 0.918$
Pancreas (Cy3/Cy5)	same time	same	$r^2 = 0.920$

Indeed, we noted that markers described in regeneration process such as Reg, PGP 9.5, and Glut 2 were upregulated. Reg mRNA was first described in 1988 (47) in regenerating pancreas. Reg is usually secreted from pancreatic acinar cells but it is also expressed in islet beta cells during regenerative processes (46). The Reg expression level in islets is positively correlated with islet cell replication and negatively associated with islet differentiation (32). An increased expression of the glucose transporter Glut2 was also described in protodifferentiated cells of the fetal pancreas (7,33) and in an experimental model of diabetes (15). Likewise, PGP 9.5, a known nerve marker, has been previously described both in fetal pancreas during development (but not after birth) and in the regeneration process following duct ligation. In these two cases, the cells with duct-like structure became PGP 9.5 positive and transitional or endocrine progenitor cells began to appear (51).

Beta and neuronal cells share a large number of similarities, the same events being able to be implicated in the differentiation of these two cell types (24). Early precursor cells to endocrine pancreas coactivate and coexpress a set of islet cell hormone and neural genes, whose expression is both selectively increased and extinguished as development proceeds. We found an upregulation of a set of neuronal genes, including tyrosine hydroxylase (TH). It has been proposed that immature beta cells may express this first enzyme of the catecholamine biosynthetic pathway that can fulfill the role of marker of islet precursor cells (38). Thus, neurotrophic factors could be in-

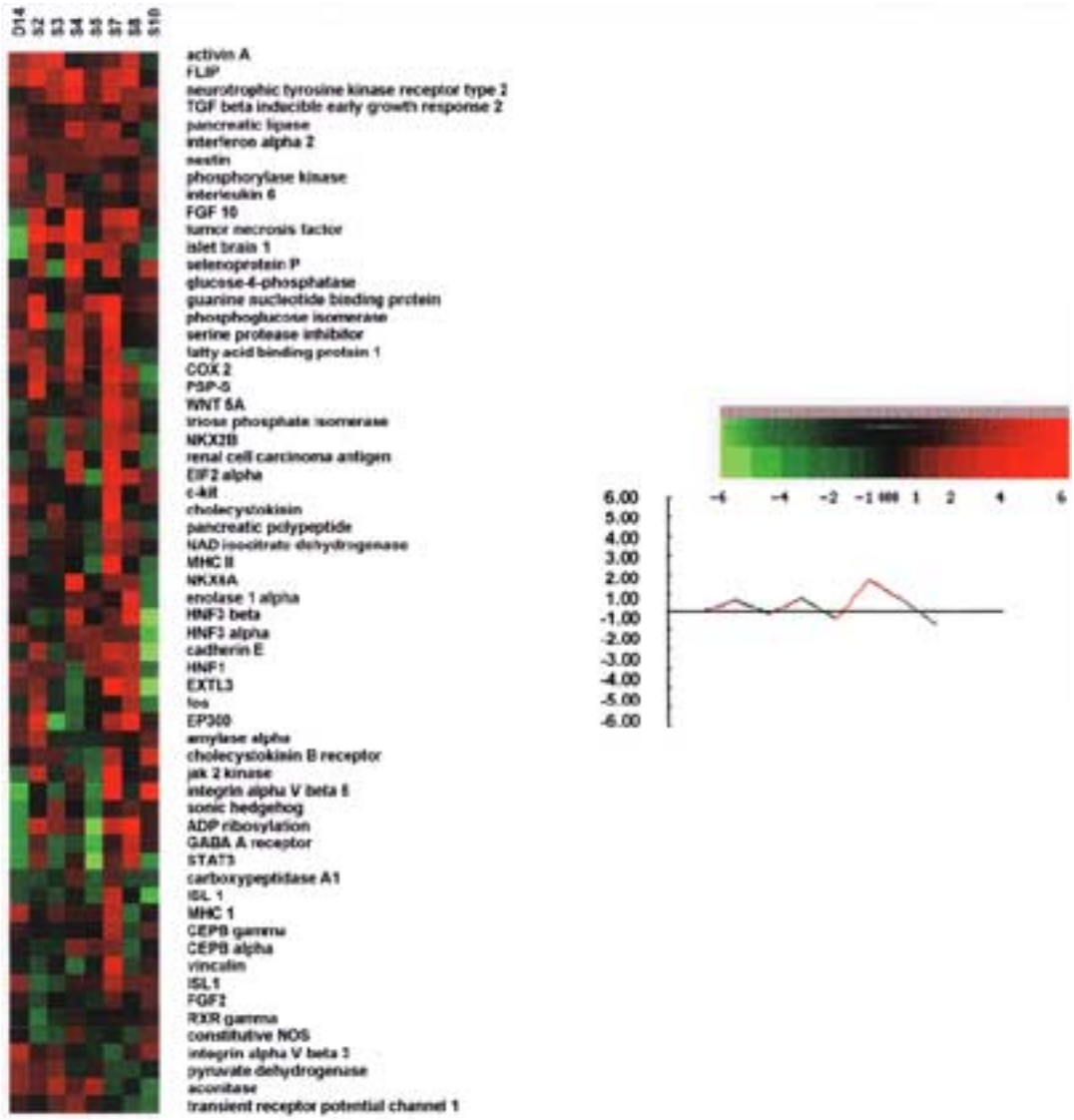

Figure 2. Genes exhibiting unchanged expression during beta cell expansion as detected by cDNA microarrays. The data are expressed as the $\log_2$ ratio (Cy5 intensity of cultured cells/Cy5 intensity of uncultured cells) of median intensity data from three individuals for each time point (day 14 of culture and subcultures S2 to S10).

volved in the development of beta cells, and TH expression may be a characteristic stage of endocrine cell precursors during maturation. TH was upregulated in our model along with other neural genes including neuropeptide Y, neuron specific enolase, and neuronatin. Studies on pancreatic cell lines have shown that neuronatin is selectively expressed by beta cells (2,31); its function may be to protect developing cells from toxic assault occurring during that period (53).

Progenitor cell markers such as FGF receptor, transferrin, and vimentin were also highly induced. FGF receptors have been identified as potentially involved in the development of endocrine pancreas (11); in particular, FGFR1-IIIb was described as a putative marker of pancreatic progenitor cells (13). Transferrin is an important growth-promoting serum glycoprotein found in many tissues of the mouse fetus, including pancreas (29), and vimentin, normally observed in the endothelial cells of blood vessels and nerves located in the adult pancreas (1), has been shown to exhibit a transient expression in the duct cells during fetal islet formation (6). This intermediate filament protein has not been observed in ducts after birth (6).

Several growth factors were upregulated, namely EGF, IGF1, FGF8, NGF TGFα, and TGFβ. This increase in growth factor production might be an at-

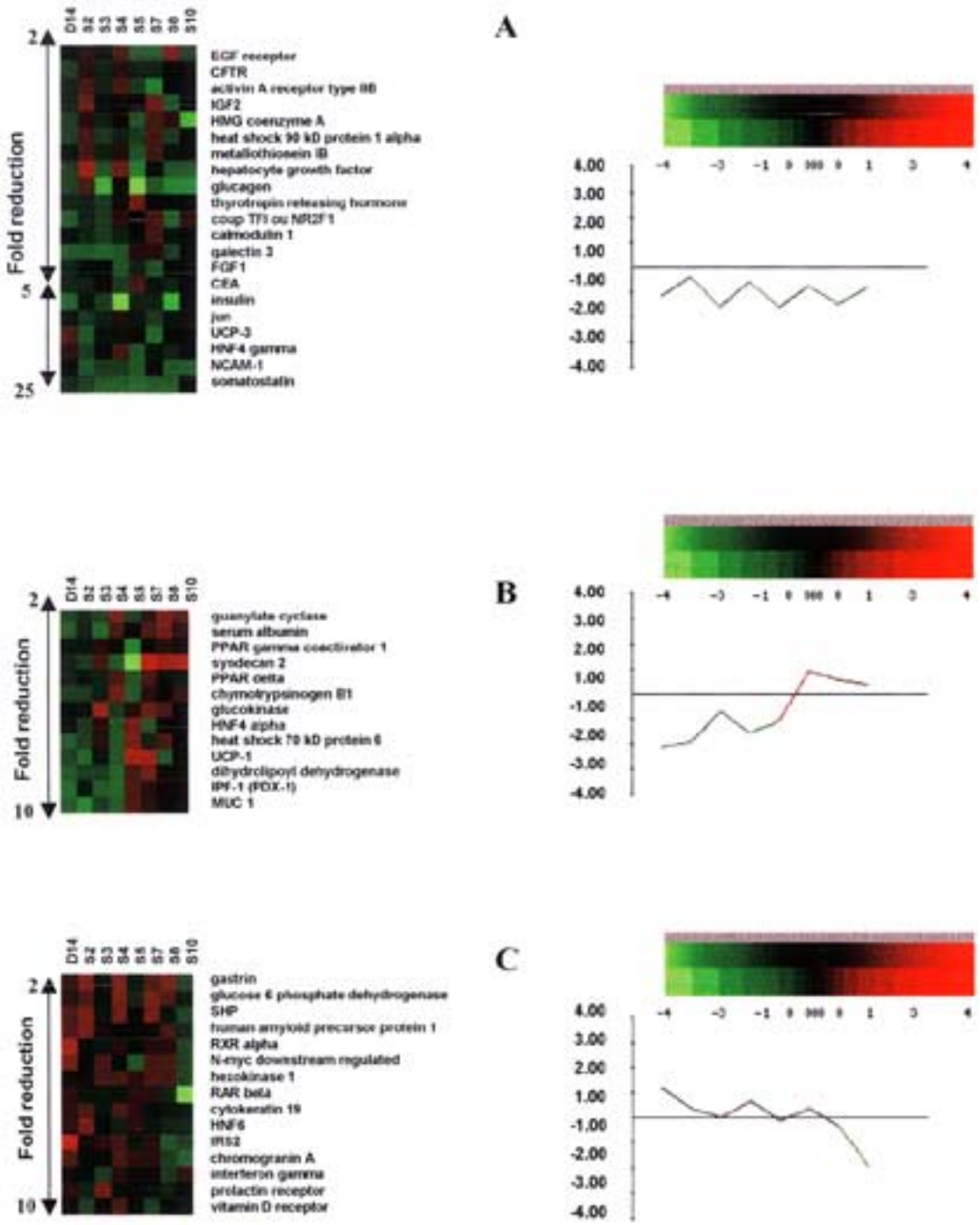

Figure 3. Genes exhibiting downregulated expression during beta cell expansion as detected by cDNA microarrays. The data are expressed as the $\log_2$ ratio (Cy5 intensity of cultured cells/Cy5 intensity of uncultured cells) of median intensity data from three individuals for each time point (day 14 of culture and subcultures S2 to S10). (A) Downregulated genes throughout the culture; (B) downregulated genes particularly in the early culture; (C) downregulated genes particularly in the late culture.

FACING PAGE

Figure 4. Genes exhibiting upregulated expression during beta cell expansion as detected by cDNA microarrays. The data are expressed as the $\log_2$ ratio (Cy5 intensity of cultured cells/Cy5 intensity of uncultured cells) of median intensity data from three individuals for each time point (day 14 of culture and subcultures S2 to S10). (A) Upregulated genes throughout the culture; (B) upregulated genes particularly in the early culture; (C) upregulated genes particularly in the late culture.

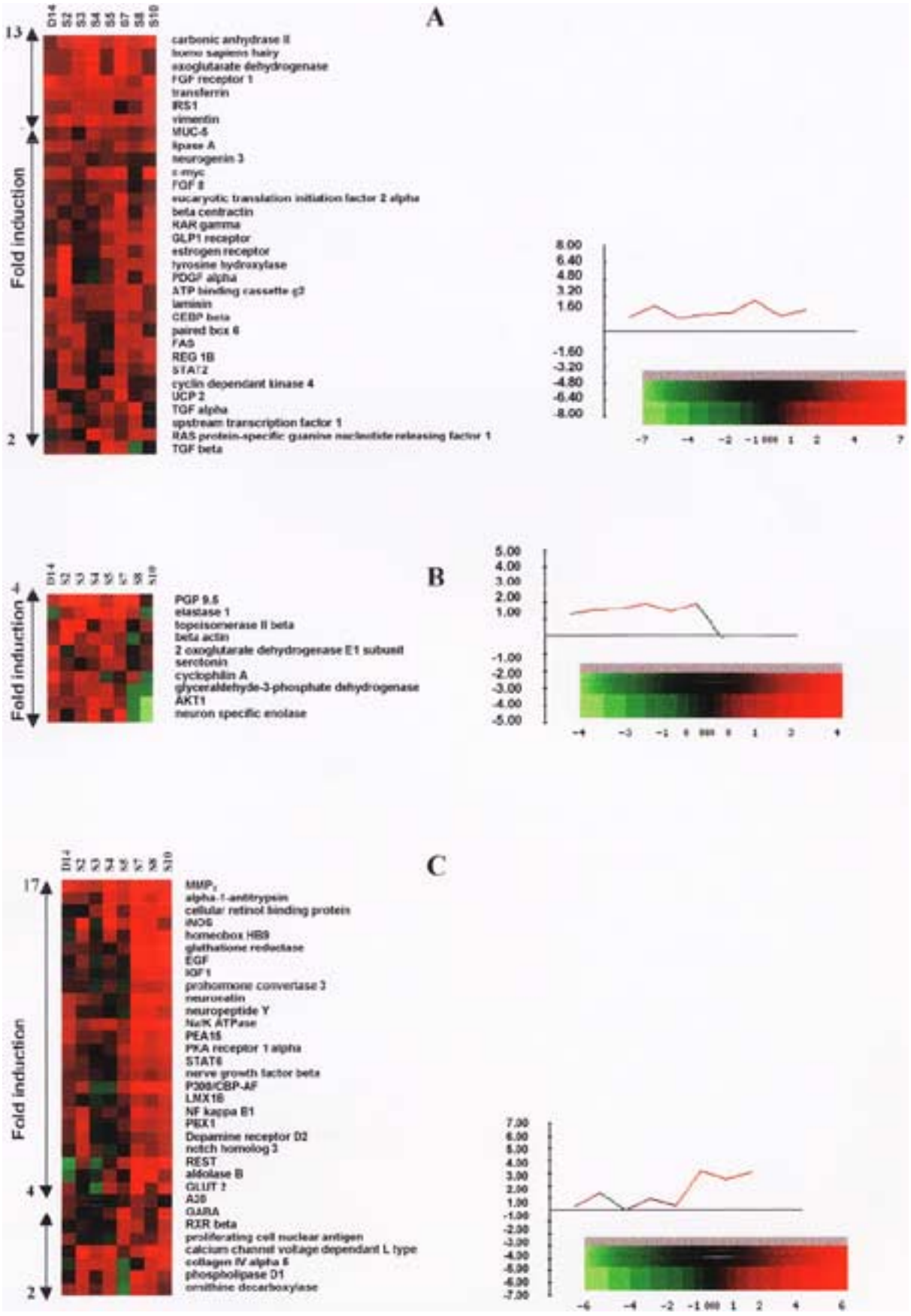
A
D14 S2 S3 S4 S5 S7 S8 S10
13
Fold induction
2
carbonic anhydrase II
homo sapiens hairy
exoglutarate dehydrogenase
FGF receptor 1
transferrin
IRS1
vimentin
MUC-6
lipase A
neurogenin 3
c-myc
FGF 8
eucaryotic translation initiation factor 2 alpha
beta centractin
RAR gamma
GLP1 receptor
estrogen receptor
tyrosine hydroxylase
PDGF alpha
ATP binding cassette g2
laminin
CEBP beta
paired box 6
FAS
REG 1B
STAT2
cyclin dependant kinase 4
UCP 2
TGF alpha
upstream transcription factor 1
RAS protein-specific guanine nucleotide releasing factor 1
TGF beta
8.00
6.40
4.80
3.20
1.60
-1.60
-3.20
-4.80
-6.40
-8.00
-7 -4 -2 -1.000 1 2 4 7
B
D14 S2 S3 S4 S5 S7 S8 S10
4
Fold induction
PGP 9.5
elastase 1
topoisomerase II beta
beta actin
2 oxoglutarate dehydrogenase E1 subunit
serotonin
cyclophilin A
glyceraldehyde-3-phosphate dehydrogenase
AKT1
neuron specific enolase
5.00
4.00
3.00
2.00
1.00
-1.00
-2.00
-3.00
-4.00
-5.00
-4 -3 -1 0.000 0 1 2 4
C
D14 S2 S3 S4 S5 S7 S8 S10
17
4
2
Fold induction
MMP2
alpha-1-antitrypsin
cellular retinol binding protein
iNOS
homeobox HB9
glutathione reductase
EGF
IGF1
prohormone convertase 3
neuroretin
neuropeptide Y
Na/K ATPase
PEA15
PKA receptor 1 alpha
STAT6
nerve growth factor beta
P300/CBP-AF
LMX1B
NF kappa B1
PEX1
Dopamine receptor D2
notch homolog 3
REST
aldolase B
GLUT 2
A2B
GABA
RXR beta
proliferating cell nuclear antigen
calcium channel voltage dependant L type
collagen IV alpha 6
phospholipase D1
ornithine decarboxylase
7.00
6.00
5.00
4.00
3.00
2.00
1.00
-1.00
-2.00
-3.00
-4.00
-5.00
-6.00
-7.00
-6 -4 -2 -1.000 1 2 4 6

TABLE 3
SEQUENCES OF PRIMERS USED FOR VALIDATION BY QUANTITATIVE RT-PCR OF RESULTS OBTAINED BY PANCREAS DNA MICROARRAY

Genes	Sense Primer (5′-3′)	Reverse Primer (5′-3′)	Size of Product (bp)
IPF-1/PDX-1	GGA GCT GGC TGT CAT GTT GAA	CGC CGC GCT TCT TGT	109
Insulin	GCA GCC TTT GTG AAC CAA CAC	CCT CGT TCC CCG CAC AC	72
PBX-1	GAT GAA TCT CCT GCG AGA GC	CTG TCG CTT GCT TGT TGA AA	200
Akt-1	GGT GAT CCT GGT GAA GGA GA	GCG TAC TCC ATG ACA AAG CA	201
Neuronatin	TGG TCT CAG CAG TTG TGG TC	AAA GTT TTG GTG CGC CTC TA	198
Ngn3	GCC CTC AAA AGC ACT TGT TC	GAA TGG GAT TAT GGG GTG GT	206
N-CAM	CGG CAT TTA CAA GTG TGT GG	GAC ATC TCG GCC TTT GTG TT	202
Chromo A	TGT AGT GCT GAA CCC CCA CC	CTC TCG CCT TTC CGG ATC TC	56
TH	GTG TTC CAG TGC ACC CAG TA	AGC GTG GAC AGC TTC TCA AT	188
RARβ	GAC CTT GAG GAA CCG ACA AA	TGG CAT TGA TCC AGG AAT TT	201
Actin	TTG CCG ACA GGA TGC AGA A	GCC GAT CCA CAC GGA GTA CT	101
18S	TCC CAG TAA GTG CGG GTC A	GAT CCG AGG GCC TCA CTA AAC	104

TH, tyrosine hydroxylase; RAR, retinoic acid receptor; chromo A, chromogranin A; N-CAM, neural cell adhesion molecule; Ngn3, neurogenin 3.

tempt of proliferating cells to adjust to culture conditions. Some of these factors are known to be phosphoinositide 3-kinase-(PI3K) or protein kinase A dependent. A major target of PI3K is the serine-threonine kinase Akt (9). Once activated, the kinases phosphorylate a number of downstream targets that usually promote cell growth and survival through regulation of protein synthesis, glycogen metabolism, and cell cycle progression (48). These two pathways were upregulated during the expansion of our cells.

The progression of mature beta cells into the cell cycle induced cell dedifferentiation. Dedifferentiation may retrace the steps followed during normal phenotype differentiation. It is worth noting that neurogenin 3 (Ngn3), a key factor for the generation of endocrine islet cells, was induced in our expanded beta cells. Ngn3 has been proposed as a marker for pancreatic islet progenitor cells in adult mice and during embryogenesis (14,20). Ngn3+ cells have transiently been detected in scattered ductal cells in the fetal mouse pancreas, peaking at embryonic day 15.5 (41). In adult human pancreatic islet cells, adenovirus-mediated delivery of Ngn3 has been shown to shift cells into neuroendocrine phenotype, with expression of insulin in a significant fraction of transdifferentiated cells (18). Ngn3+ cells can thus play a role in the postnatal growth of pancreatic beta cell mass. In our model of dedifferentiation, Ngn3 gene expression was upregulated as early as day 14 of culture and remained overexpressed throughout the culture period; Ngn3 protein expression was obvious from subculture 4 on, thus showing the potent ability of expanded cells to revert to endocrine cells provided adequate conditions are met. We have previously shown that one of the possible conditions could be sodium butyrate application, which induced in expanded cells a reexpression of insulin and IPF-1/PDX-1 and an increase in insulin content as well as activin A application, which enhanced insulin secretion (5). Interestingly, using AR42J-B13 cells as a model of the differentiation of pancreatic islets, Ogihara et al. (30), have recently shown that activin A and HGF negate the repressor activity of a region between −402 and 327 bp of Ngn3 gene promoter. Cell differentiation induced by sodium butyrate could likewise be related to deacetylase inhibition, leading to hyperacetylation of chromatin components that results in activation of gene expression or abolition of gene repression (35).

To summarize, multiple investigators have shown that tissue-specific stem cells, intermediate precursors, and even fully differentiated postmitotic cells can be induced to alter their phenotypic profile (43). This can be achieved by cell proliferation that induces transdifferentiation and occurrence of multipotential dedifferentiated intermediate cells that express markers characteristic of several alternative phenotypes (40,52). Cells may thus follow multiple pathways to acquire a distinct phenotype and may also revert to an earlier, more primitive phenotype that would have a wider differentiation potential. Our study may contribute toward depicting progenitor cell features occurring during culture of human pancreatic beta cells, to provide essential tools to direct in vitro differentiation of transplantable insulin-secreting cells from precursor cells. This knowledge may also be useful in the design of treatments aiming at stimulating pancreatic beta cell regeneration in vivo.

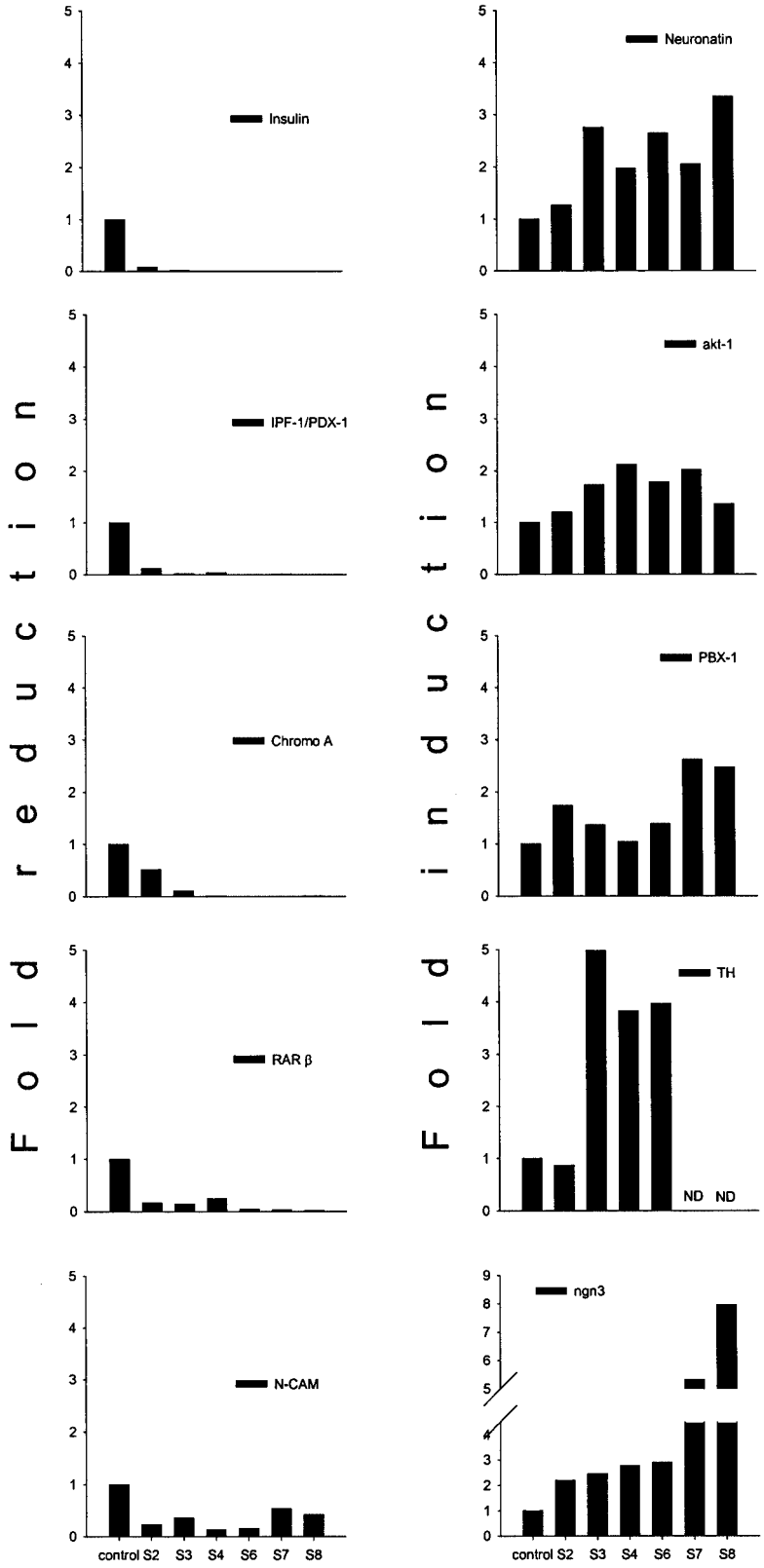

Figure 5. Gene expression during beta cell expansion assessed by quantitative real-time RT-PCR as described in Materials and Methods. Values of cultured cells were corrected with respect to values of uncultured cells arbitrarily set at 1. ND: not determined. TH: Tyrosine hydroxylase, RAR: Retinoic acid receptor, Chromo A: Chromogranin A, N-CAM: Neural cell adhesion molecule, Ngn3: Neurogenin 3.

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