Functional characteristics of neonatal rat β cells with distinct markers

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Abstract

Neonatal β cells are considered developmentally immature and hence less glucose responsive. To study the acquisition of mature glucose responsiveness, we compared glucose-regulated redox state, insulin synthesis, and secretion of β cells purified from neonatal or 10-week-old rats with their transcriptomes and proteomes measured by oligonucleotide and LC-MS/MS profiling. Lower glucose responsiveness of neonatal β cells was explained by two distinct properties: higher activity at low glucose and lower activity at high glucose. Basal hyperactivity was associated with higher NAD(P)H, a higher fraction of neonatal β cells actively incorporating ³H-tyrosine, and persistently increased insulin secretion below 5 mM glucose. Neonatal β cells lacked the steep glucose-responsive NAD(P)H rise between 5 and 10 mM glucose characteristic for adult β cells and accumulated less NAD(P)H at high glucose. They had twofold lower expression of malate/aspartate-NADH shuttle and most glycolytic enzymes. Genome-wide profiling situated neonatal β cells at a developmental crossroad: they showed advanced endocrine differentiation when specifically analyzed for their mRNA/protein level of classical neuroendocrine markers. On the other hand, discrete neonatal β cell subpopulations still expressed mRNAs/proteins typical for developing/proliferating tissues. One example, delta-like 1 homolog (DLK1) was used to investigate whether neonatal β cells with basal hyperactivity corresponded to a more immature subset with high DLK1, but no association was found. In conclusion, the current study supports the importance of glycolytic NADH-shuttling in stimulus function coupling, presents basal hyperactivity as novel property of neonatal β cells, and provides potential markers to recognize intercellular developmental differences in the endocrine pancreas.

Key Words

- islet cells
- microarray
- neonatal
- pancreatic β cell
- gene expression

Introduction

Therapeutic control of β cell proliferation is considered an important route toward cure in diabetes. The interest in the underlying mechanisms and signals that control β cell proliferation has revived the interest in the physiological conditions characterized by activated β cell growth and/or proliferation. Examples include the physiological β cell mass expansion during pregnancy, in adaptation to obesity and during growth. Here, we studied the β cell in
the neonatal pancreas (2–3 days after birth): during the first months of postnatal rat life, the total β cell number expands more than tenfold mainly due to β cell division (Hellmann 1959, Hellman et al. 1961, McEvoy 1981, Kaung 1994). Fetal and neonatal β cells have also been reported to have a lower glucose-stimulated insulin secretion (GSIS) than adult β cells (Asplund et al. 1969, Grill et al. 1981, Hellerstrom & Swenne 1991). Both their higher propensity to proliferate and their lower functional glucose responsiveness are generally considered as two sides of a same coin: both properties are assumed to reflect glucose responsiveness are generally considered as two sides of a same coin: both properties are assumed to reflect glucose responsiveness and need to be accounted for when considering potential protein markers for β cell maturation. A recent study investigated a number of putative markers that purported to distinguish between mature and immature β cells (Grill et al. 2010). In this study, we sought to identify novel protein markers for β cell maturation and acquisition of glucose responsiveness, by comparing the mRNA and protein expression blueprint and glucose-regulated insulin synthesis and secretion of neonatal (postnatal days 2–3) and adult (10 weeks old) rat β cells. More specifically, our aim was dual: i) identify positive markers of differentiation by studying the postnatal appearance of the gene expression pattern characteristic for mature, fully differentiated β cells and ii) investigate possible markers for the presumed residual developmental immaturity in the neonatal stage, and see whether their expression correlates with the typical functional properties of the neonatal β cells. We describe a FACS-based method to isolate a representative population of β cells from rat pancreas at postnatal days 2–3. Their gene expression and in vitro function was compared with those of 10-week-old adult β cells, serving as reference. As anticipated, neonatal β cells displayed a lower glucose-inducible insulin synthesis, but surprisingly, this was not only explained by a blunted response to high glucose stimulation but also explained by basal hyperactivity at low glucose concentrations. This led to further investigation into the differential gene expression between neonatal and 10-week-old FACSSorted rat β cell preparations to get a global view on endocrine differentiation in neonatal β cells and to select a possible protein marker for residual developmental immaturity at this stage.

**Materials and methods**

**β cell isolation and culture**

Adult (10 weeks) male Wistar rats (150–250 g, Janvier, Le Genest Saint Isle, France) were bred according to Belgian regulations of animal welfare and used in experiments that were approved by the Local Ethics Committee. Use of these animal cells and tissues was approved by the Commissie Proefdierengebruik (CPG, VUB for a project entitled ‘in vitro and in vivo markers for β cell death and function’, approval ID 07-274-3). Ten-week-old (adult) β cells were FACS purified as described in Van De Winkel & Pipeleers (1983). Neonatal β cells were isolated from male and female rats at postnatal days 2–3 as follows (Fig. 1): i) neonatal endocrine aggregate-enriched preparations were collected by gradient centrifugation rather than handpicking of islets in 10-week-old rats: collagenase-digested pancreas was density centrifuged first over a Ficoll layer (1.10 g/ml) to separate exocrine from endocrine cells; the endocrine-enriched interphase was collected and ii) trypsinized to single cells, additionally purified over a Percoll (1.04 g/ml) layer to separate viable from damaged cells prior to FACS sorting. The 10-week-old β cells are mainly discriminated from other islet cell types by their higher cell size (forward scatter (FSC)) in combination with higher FAD/FMN fluorescence (Fig. 1C; Van De Winkel & Pipeleers 1983). As their flavin fluorescence is lower in neonatal β cell, a combination of FSC and cellular complexity/granulation (side scatter (SSC)) is used to separate neonatal β cells from a less granulated cell population; the latter SSCLOW population contains not only exocrine duct cells but also endothelial and mesenchymal cells and a very small fraction of insulin-positive cells.

**In vitro experiments**

Rat β cells were cultured in Ham’s F10 nutrient mixture (Gibco, Invitrogen Corporation) supplemented with 0.5% BSA (Cohn Analog, Sigma), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at the indicated glucose concentrations. Protein biosynthetic rates were measured by 3H-Tyr incorporation, in batch analysis, or in single cells on slides by autoradiography as described previously (Kiekens et al. 1992). Analysis of metabolic redox state (NAD(P)H, FAD/FMN) was done as described...
All chemicals were obtained from Sigma unless otherwise stated. Gene chip hybridization and data analysis

One to three micrograms of RNA were extracted from three independent biological replicates, each consisting of a β cell pool obtained from ten 10-week-old or 50 neonatal rats, and cRNA hybridized to Affymetrix (Santa Clara, CA, USA) RG230.20 arrays as described previously (Martens et al. 2011). Raw data and MIAME are shared at Gene Expression Omnibus as data set GSE47174. Gene chip data analysis: scanned arrays were analyzed with dChip model-based expression analysis (Li & Wong 2001).
Table 1  Quantitative LC-MS/MS proteomics of neonatal and 10-week-old β cells. Total cellular protein was extracted from freshly isolated neonatal (n=4) and 10-week-old (n=3) β cells, trypsinized, and analyzed by one-dimensional liquid chromatography alternate-scanning mass spectrometry. This allows precise relative quantification of the molar abundances of identified protein within one cell preparation. These relative molar abundances were then expressed as fold change of the corresponding cyclophilin A/peptidylprolyl isomerase A (PPIA) abundance of that same preparation as reference, to allow normalization across different cell types and isolations. Data indicate average ± s.d. of target/PPIA relative molar amounts of the selected proteins.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein description</th>
<th>Uniprot</th>
<th>Neonatal β</th>
<th>10-week rat β</th>
</tr>
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<tr>
<td>Glycolysis</td>
<td></td>
<td></td>
<td>(average ± s.d.)</td>
<td>P</td>
</tr>
<tr>
<td>Gpi</td>
<td>Glucose 6 phosphate isomerase</td>
<td>Q6P6V0</td>
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<td>Aldoa</td>
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<td>Tpi</td>
<td>Triosephosphate isomerase</td>
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<td>Pgk1</td>
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<td>Pgam1</td>
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<td>Pklr</td>
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<td>Malate–aspartate</td>
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<td>NADH shuttle</td>
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<tr>
<td>Mdh1</td>
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<td>Q89899</td>
<td>2.219 ± 0.052</td>
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<td>Aspartate aminotransferase cytoplasmic</td>
<td>P13221</td>
<td>0.086 ± 0.009</td>
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<td>Echdc1</td>
<td>Enoyl CoA hydratase domain containing protein 1</td>
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<td>Protein synthesis</td>
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<tr>
<td>Eef2</td>
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<td>P05197</td>
<td>0.360 ± 0.023</td>
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<td>Eef1a1</td>
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<td>Protein disulfide-isomerase A3</td>
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<td>Q63081</td>
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<td>Q00981</td>
<td>0.396 ± 0.010</td>
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after normalization to array with median intensity (default) and using background correction for mismatch hybridization. dChip was also used for hierarchical clustering (correlation/centroid mode by cluster tightness) and identification of statistically ($P < 0.005$) enriched gene ontologies.

LC-MS/MS proteomics

Peptide identification, and quantitative data analysis were performed exactly as described (Martens et al. 2010, Jiang et al. 2013). In brief, using one-dimensional liquid chromatography followed by alternate-scanning tandem mass spectrometry, we compared three biological replicates of 10-week-old β cells with four biological replicates of neonatal β cells. Each biological replicate represents 0.5–1 μg total protein, extracted using Rapigest (Waters Corp., Milford, PA, USA) detergent from an unfractionated β cell pool obtained from 15–30 individual rats, resulting in 296 (469) protein identifications in neonatal (10-week-old) β cells; a selection of relevant gene ontologies is presented in Table 1. A table listing all identifications is available on request. Relative molar amounts for individual LC-MS/MS runs were calculated as described previously (Silva et al. 2006, Martens et al. 2010); comparison between different cellular isolates was done after normalization toward cyclophilin-A/PPIA ($Ppia$ gene). PPIA was selected as a reference protein as in all neonatal and 10-week-old β cell preparations; the PPIA ratio to other housekeeping proteins such as EEF1A1, HSPA8, MDH2, PFN1, TUBB5, and YWHAE showed coefficients of variations (CV) <35%.

Real-time PCR analysis of rat β cells and tissues

Total RNA was extracted from pancreatic endocrine cells using RNAeasy (Qiagen) minicolumns, according to the manufacturer’s protocol. RNA quality was verified by Agilent Bioanalyzer (cutoff RIN ≥ 7; Santa Clara, CA, USA). Following removal of genomic DNA (TURBO DNA-free, Ambion, Austin, TX, USA) and RT (High-Capacity cDNA Archive Kit, Applied Biosystems), targets were amplified from cDNA template on ABI Prism 7700 Sequence Detector using TaqMan Universal PCR Master
Mix and commercially available sequence-specific primers and TaqMan MGB probe (Applied Biosystems, assays’ IDs available on request). Also for PCR, Ppia was validated as reference for relative quantification based on its stable expression ratio (CV < 35%) toward mRNA level of several other classical reference genes (Ubc, Hprt1, and Psme5) in all neonatal and adult β cell isolates (data not shown), as its absolute mRNA abundance was in the range of Ct values of most target mRNAs. β-actin (Actb) was also invalid as reference for PCR normalization as neonatal β cells expressed four times ($P<0.01$, $n=7$) more Actb mRNA levels than adult cells, as normalized to Ppia and Ubc.

**Immune stainings**

Non-specific staining was blocked with 10% donkey serum in PBS followed by incubation overnight at 4°C with primary antibodies, 60 min with secondary antibodies and addition of bisbenzimide (Hoechst 33342, Sigma). Antibody provenance is as follows: anti-insulin (guinea pig, 1:5000, in house, C Van Schravendijk), anti-glucagon (rabbit, 1:2000, in house), anti-delta-like 1 homolog (pig, 1:5000, in house, C Van Schravendijk), anti-glucagon (rabbit, 1:2000, in house), anti-delta-like 1 homolog (pig, 1:5000, in house, C Van Schravendijk), anti-glucagon (rabbit, 1:2000, in house). Secondary antibodies, raised in donkey, were obtained from Jackson Immunoresearch (Suffolk, UK). Staining was typically done on paraffin-embedded tissue after EDTA-antigen retrieval. B6.FVB-Tg(Npy-hrGFP)1Low mice expressing humanized Renilla Green Fluorescent Protein (hrGFP, Stratagene, Agilent) under control of the mouse neuropeptide Y (Npy) promoter were generated by B Lowell (Beth Israel Deaconess Med Center, Harvard, USA) and distributed by The Jackson Laboratory (stock number 006417; Bar Harbor, ME, USA). Secondary antibodies, raised in donkey, were obtained from Jackson Immunoresearch (Suffolk, UK). Staining was typically done on paraffin-embedded tissue after EDTA-antigen retrieval. B6.FVB-Tg(Npy-hrGFP)1Low mice expressing humanized Renilla Green Fluorescent Protein (hrGFP, Stratagene, Agilent) under control of the mouse neuropeptide Y (Npy) promoter were generated by B Lowell (Beth Israel Deaconess Med Center, Harvard, USA) and distributed by The Jackson Laboratory (stock number 006417; Bar Harbor, ME, USA). hrGFP fluorescence was recorded on cryosections of freshly isolated pancreas tissue. Images were acquired on Axioplan (Carl Zeiss) microscope using Smartcapture VP Software; digital processing was limited to a 5 × 5 soft enhance filter. Correlation of autoradiography with selected protein markers was done using an in-house developed algorithm (G Stange): pictures were made with a Nikon Eclipse Te2000 inverted fluorescence microscope. TIF files were imported into Attovision for segmentation of the cells on the basis of their DAPI/Insulin/DLK1 intensity. The number of silver granules, representing incorporated $^{3}$H-tyrosine, was counted within the region of interest of individual cells. All data were exported as flow cytometry standard (FCS) file and further processed with FlowJo (Treestar, Ashland, OR, USA).

**Statistical analysis**

Statistical differences were assessed using two-tailed unpaired T-test, assuming unequal variances unless otherwise specified.

**Results**

**Cellular composition and structural characteristics of the isolated neonatal β cell fractions**

Endocrine-enriched fractions obtained by density gradient centrifugation of dissociated pancreas from postnatal day 2–3 rats were FACS sorted in cell fractions with high (SSC$^{\text{HIGH}}$) and low (SSC$^{\text{LOW}}$) SSC (Fig. 1A). SSC$^{\text{HIGH}}$ cells contained ± 90% cells with endocrine ultrastructure on electron microscopy, composed of 67 ± 6% insulin- and 19 ± 5% glucagon-expressing cells. The non-granulated SSC$^{\text{LOW}}$ cells were not further studied; they likely represent a mixed population containing few insulin-expressing cells, but predominantly cells expressing mRNA markers of endothelial or mesenchymal cells and pancreatic ductal epithelium (Fig. 1B, $n=15$). Around 15 ± 6 × 10$^{3}$ insulin-expressing cells per neonate were isolated in the SSC$^{\text{HIGH}}$ window. As 6 ± 3% of insulin-positive cells was recovered in the less granulated SSC$^{\text{LOW}}$ fraction (Fig. 1A), these SSC$^{\text{HIGH}}$ insulin-positive cells are further designated as ‘neonatal β cells’. They were compared with a reference cell preparation of 88 ± 3% insulin purity, FACS purified from 10-week-old rats (Fig. 1C and D). The fraction of all endogenous β cells that could be isolated and studied further in vitro was similar in neonates and 10-week-old rats and amounted to ± 10% of total β cell number contained in the whole pancreas (Chintinolle et al. 2010).

Electron microscopy indicated a 32% higher cross-sectional surface of 10-week-old than neonatal β cells ($P<0.005$, Supplementary Table 1, see section on supplementary data given at the end of this article). As their nuclear radius was similar ($r=2.9±0.2\ \mu m$), 10-week-old β cells (1400 $\mu m^{3}$) thus had a 1.6 times higher estimated cytoplasmic volume than neonatal β cells (900 $\mu m^{3}$). Adult (44 ± 4 pg ins/cell, $n=5$) and neonatal (41 ± 5 pg ins/cell, $n=5$) β cells did not differ in their cellular insulin stores.

**Glucose-regulated insulin synthesis and secretion and metabolic redox state of neonatal β cells: higher activity at low glucose and lower activity at high glucose**

We compared glucose-inducible protein biosynthesis and secretion by neonatal and 10-week-old β cells, during
short-term (1 h) exposures immediately upon cell isolation and during more sustained cultures of 24 h (Table 2, n=4). Neonatal β cells displayed two main differences compared with 10-week-old cells: first, neonatal β cells synthesized ±2 times less protein (P<0.005) under high glucose (20 mM) stimulation than 10-week-old cells, and, secondly, they synthesized up to seven times more protein at basal glucose concentrations (2.5 mM). The latter basal hyperactivity appeared to be a stable property of the neonatal β cells: i) it was preserved also after 24-h of in vitro exposure (Table 2) and ii) it could also be measured in islets isolated from animals up to 5 weeks old but not older, isolated using the same protocol (Supplementary Figure 1, see section on supplementary data given at the end of this article). Together, both properties account for the markedly lower (5.6-fold, P<0.005) glucose-inducible protein synthesis of neonatal β cells, when defined as the increase in molar insulin output per cell by raising glucose concentration from 2.5 to 10 mM.

To analyze whether basal hyperactivity was a uniform characteristic of all neonatal β cells, protein synthetic activity of individual insulin-positive cells was measured by 3H-Tyr autoradiography. A β cell was considered to be recruited into a state of activated protein synthesis when it contained ≥30 3H-tyrosine-labeled secretory granules per cell after 30-min exposure to 0–10 mM glucose (Fig. 2A).

Neonatal β cells contained a higher fraction of cells with

![Table 2](image-url)

Comparison of insulin biosynthesis and secretion of newly synthesized insulin in neonatal and 10-week-old β cells. β cells were isolated from 1-day-old neonatal and 10-week-old pancreas and compared for their rates of 3H-tyrosine incorporation in the protein fraction immunoprecipitated with anti-insulin antibody. Incorporation of the 3H-tyrosine label was measured in the intracellular insulin fraction (synthesized intracellular) and in the insulin fraction released into the culture medium (synthesized secreted) over 1- and 24-h incubations at the indicated glucose concentrations. Synthesized total represents sum of newly synthesized intracellular and secreted insulin. Insulin output per β cell was corrected for the insulin-positive purity, measured in each individual preparation and averaging 90% in 10-week-old and 70% in neonatal β cell preparations. No correction for smaller β cell size of neonatal β cells was made. Right panel shows a calculation of the insulin synthetic and secretion rates per hour of the 24-h incubations. Data represent mean ± s.d. of duplicate measurements on n=4 independent isolations.

<table>
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<tr>
<th>Glc (mM)</th>
<th>Synthesized intracellular</th>
<th>Synthesized secreted</th>
<th>Synthesized total</th>
<th>Fractional secretion</th>
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<tr>
<td></td>
<td>β cells per h</td>
<td>β cells per h</td>
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<tr>
<td></td>
<td>Mean ± s.d.</td>
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<td>P10-week vs neo</td>
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<td>1-h incubation (fmol insulin/10⁴ β cells per h)</td>
<td>24-h incubation (fmol insulin/10⁴ β cells per h)</td>
<td>Insulin output/h (24 vs 1 h incubations)</td>
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<tr>
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<td>2.5</td>
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<tr>
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P value were calculated by unpaired Student's t-test assuming equal variances (n=4). Insulin outputs are expressed per 10⁴ β cells following correction for insulin-positive purity but not cell size.
activated protein synthesis, when compared with the 10-week-old β cells (Fig. 2B); even in the absence of glucose, half of neonatal β cells showed intense insulin synthetic activity and raising glucose concentration to only 2.5 mM recruited an additional 30% of cells. To achieve a similar fraction of biosynthetically active cells as neonatal cells at 2.5 mM, 10-week-old β cells required more than 10 mM glucose. Glucose exerted a dual effect both in neonatal and in 10-week-old β cells: apart from the concentration-dependent recruitment of β cells into active protein synthesis (Fig. 2A and B) also an additional amplyfying effect in the recruited cells was also observed. Although also present in neonatal cells, the latter amplification appeared much stronger (Table 2) in 10-week-old β cells, leading to a three- to fourfold higher maximal synthetic capacity per active β cell at 10 weeks. Of note, the basal protein synthetic hyperactivity in neonatal β cells was due to increased insulin synthesis with no difference between young/older β cells in the amount of non-insulin protein produced (Supplementary Figure 2, see section on supplementary data given at the end of this article).

As insulin production rate in adult β cells is stoichiometrically correlated with nutrient oxidation rates (Martens et al. 2006), we examined whether this was also the case in neonatal cells: as an indicator for glucose metabolic rate, we measured acute nutrient-induced NAD(P)H accumulation (Fig. 2C and D) and FAD/FMN reduction to FADH2/FMNH2 (Supplementary Figure 2, n = 4). In line with their mature secretory competence, 10-week-old β cells showed clear nutrient-responsive NAD(P)H fluctuations: NAD(P)H increased sharply by raising glucose from 5 to 10 mM, by supplementing 5 mM glucose with equivalent amounts of mitochondrial nutrients α-glyceraldehyde (1 mM) or methylsuccinate (10 mM) or by blocking NADH consumption by complex I inhibitor rotenone. Despite their smaller size, neonatal β cells tended to have higher NAD(P)H fluorescence at glucose ≤ 5 mM and this could not be further increased, neither by mitochondrial nutrients nor by rotenone, suggesting that they were already leveled at a plateau phase. FAD/FMN reduction showed similar trend although less discriminating (Supplementary Figure 2). Mannoheptulose blocked 10 mM glucose-induced NAD(P)H (Fig. 2D) and protein synthesis (Supplementary Figure 2C) at both ages, but only in neonatal β cells, it partly suppressed insulin synthesis (P < 0.05) and NAD(P)H (P = 0.10) at low glucose.

Neonatal β cells also secreted more insulin at low glucose, although this effect was modest when compared with their lower secretion at high glucose: yet, this basal secretory hyperactivity at glucose ≤ 5 mM was detected both in short-term (1 h) exposures and in more sustained exposures up to 3 days (Table 2, Fig. 2C, D, E and F, P < 0.05). Neonatal β cells were less prone to glucose-induced insulin degranulation in culture (Fig. 2D and E), in part, because they secrete a lower fraction of their insulin content under high glucose stimulation (P < 0.05).

Advanced endocrine differentiation of neonatal β cells as judged by their mRNA level of conserved β cell marker genes

Lower glucose responsiveness of insulin release by neonatal β cells was previously attributed to a generalized immaturity of the specialized β cell phenotype detectable by Affymetrix oligonucleotide 230.20 array (Jermendy et al. 2011). We used the same platform to compare the transcriptome of freshly isolated neonatal and 10-week-old rat β cells. Considering the 30% higher insulin purity of 10-week-old β cells, a threshold of twofold (lower confidence bound (LCB), P < 0.05, n = 3) was selected as minimum for differential expression. Of all assayed transcripts, 2.3% (Supplementary Table 2, see section on supplementary data given at the end of this article) matched these criteria: 443 transcripts were upregulated in neonatal β cells and 270 transcripts were higher in the adult β cells. The 270 transcripts upregulated in 10-week-old β cells were expected to reflect postnatal maturation of the specialized β cell phenotype with anticipated increase in gene expression of key regulators of endocrine function. This was not the case:

First, the genes activated in adult β cells showed no statistical enrichment in gene ontologies relating to nutrient-sensing, regulated hormone synthesis or secretion or any other indicator of specialized neuro-endocrine function. It showed statistical enrichment (P < 0.001) of gene clusters involved in exocrine pancreatic function (peptidases, ribonuclease, triacylglycerol lipase activities, and zymogen granule components; Supplementary Figure 4, see section on supplementary data given at the end of this article), indicating more exocrine contamination. Electron microscopy showed low overall exocrine contamination, but a tendency for more in the 10-week-old (2%, Fig. 1B, C and D) than in the neonatal (1%, P = 0.45) β cells.

Secondly, we specifically analyzed mRNA expression of key β cell marker genes, using a previously validated panel of 419 probe sets on Affymetrix RG230.20,
Figure 2
Basal hyperactivity of neonatal β cells at level of protein synthesis, cellular NAD(P)H, and insulin secretion. (A) Autoradiographic measurement of 3H-tyrosine incorporation in secretory vesicles (white dots) in insulin-positive cells (red fluorescence) at 2.5 and 10 mM glucose. Scale bar, 10 μm. (B) Quantification of insulin-positive cells (mean ± s.d., n = 5 independent isolates, > 500 cells counted/condition) with > 30 labeled secretory granules after 1-h labeling in vitro at the indicated glucose concentration in neonatal (gray squares) and 10-week-old (black circles) insulin-positive cells. (C and D) Mean fluorescence intensities of total cellular NAD(P)H as measured by FACS, in freshly isolated, propidium iodide-negative neonatal/10-week-old β cells, incubated for 1 h at the indicated nutrient/inhibitor concentrations. For simultaneously recorded FAD/FMN fluorescence, see Supplementary Figure 2. Bars represent geometric average of 4–5 isolations. (E, F and G) The static insulin secretion of aggregated neonatal (gray bars) and 10-week-old (black bars) β cells during 64 h after isolation with medium refreshment every 24 h: by measuring amount of insulin secreted over 64 h (E), and measurement of cellular insulin content at start and end of culture (F) and at 40 h (not shown), fractional excretion can be calculated (G). Bars represent mean ± s.d., n = 5, *P < 0.05 (unpaired Student’s t-test) neonatal vs 10-week-old β cells.
corresponding to 332 unique genes with a conserved β cell-selective expression pattern (Martens et al. 2011). Less than 2% of these conserved β cell marker genes were twofold (LCB) differentially expressed in neonatal and 10-week-old β cells and <10% when the threshold was lowered to 1.5-fold (LCB) (Fig. 3A). Gene expression of 22 β cell markers was verified by real-time PCR. Only for Npy and St18, the microarray and real-time PCR results were congruent. Relative to their Ppia (and Ubc, not shown) mRNA levels, neonatal β cells expressed similar levels of Iapp, Dcx, or Gad2 and even markedly higher levels of several other β cell marker genes such as Ins2, Pdx1, Hadh, Nkx6.1, and Ptprn/Ia-2 and glucokinase than adult β cells (Fig. 3A and B, n = 4). In view of their different metabolic redox properties, we additionally compared both age groups for differences in mRNA expression of enzymes involved in the metabolism/use of NAD(P)+ and FAD/FMN cofactors (Supplementary Figure 3, see section A).

Figure 3
Neonatal rat β cells show advanced differentiation toward the specialized β cell phenotype. (A) Compares neonatal and 10-week-old β cells on Affymetrix RG230.20 platform for their mRNA expression of a previously validated panel of 332 genes with conserved β cell-selective expression (Martens et al. 2011). Even using a relatively loose statistical limit of 1.5-fold difference, only 10% of these probe sets were differentially expressed, corresponding to 31 non-redundant genes shown on the right of (A): blue to red indicate low to high mRNA signal on gene chip respectively; genes are indicated by gene symbol, full name, and NCBI accession. Fold change (lower confidence bound (LCB)) of neonate/10-week-old cells is shown with P value (n = 3) corrected for multiple comparisons. Plus and minus signs in last column indicate successful or failed confirmations by real-time PCR in (B). (B) mRNA expression levels measured by TaqMan hydrolysis-probe PCR of a panel established and more recently reported (St18, Wnt4, and Dcx) β cell marker genes. mRNA levels are expressed as percent of Ppia mRNA level in neonatal (black bars) or 10-week-old (gray bars) β cells (*P < 0.05, n = 3–5, bars represent mean ± S.D.).
on supplementary data given at the end of this article. Here again, differences were minimal. One gene stood out: malate dehydrogenase 1 (Mdh1); the cytoplasmic arm of the malate/aspartate NADH shuttle had a sevenfold higher mRNA level in adult β cells, in line with previous observations (Tan et al. 2002, Jermendy et al. 2011).

Metabolic and endocrine differentiation as measured by quantitative LC-MS/MS proteomics

We analyzed unfractionated proteomes of neonatal rat β cells (n=4), as was done previously for 10-week-old rat β cells (n=3) (Martens et al. 2010), and compared relative molar abundances after normalization to PPIA protein levels. Table 1 highlights protein expression of key enzymes of intermediary nutrient metabolism, various proteins involved in hormone synthesis and secretion, and classical endocrine vesicular constituents. This analysis indicates that acquisition of mature glucose responsiveness associates with a comprehensive twofold upregulation of glycolytic enzymes and the cytoplasmic arm of the malate/aspartate NADH-shuttle, notably MDH1. Neonatal and 10-week-old β cells show no marked differences in their expression of various mitochondrial metabolic pathways nor do they differ in their expression of key enzymes involved in protein translation, folding, and vesicle secretion. Quite remarkably, neonatal β cells showed two- to threefold higher protein levels of many classical intravesicular proteins, not only of the secretogranin family but also including key hormone-processing enzymes PCSK1 and CPE, often considered as cellular markers of β cell maturation.

Candidate markers for residual developmental immaturity

Four hundred and forty three transcripts showed twofold (LCB, P<0.05, n=3) higher expression in neonatal than in 10-week-old β cells. These genes were statistically (P<0.001) enriched in gene ontology clusters such as cell cycling, DNA replication, extracellular growth factor signaling, and cell growth (exemplary genes shown in Fig. 4).

Eight genes were successfully confirmed by real-time PCR (Fig. 4, n=5): these included several novel candidates (Gap43, Nrep, Egln3, and Racgap1) that were strongly (5- to 25-fold, P<0.05, n=5) downregulated upon β cell maturation, as well as several genes that were previously reported to mark more immature β cell stages, and thus serve as positive controls: Dlk1 and Pref1 (Carlsson et al. 1997), Mafb (Nishimura et al. 2006), thyrotropin-releasing hormone (Trh) (Martino et al. 1978, Dolva et al. 1983), and Npy (Myersen-Axcrona et al. 1997). Npy mRNA was 5±1 times (P<0.05, n=5) higher in neonatal β cells but could still confidently be detected in 10-week-old β cells. By contrast, Mafb and Dlk1 mRNA could not be consistently amplified after 40 PCR cycles in five independent isolates of 10-week-old β cells and consequently showed most massive downregulation. In neonatal β cells, Dlk1 mRNA showed exceptionally high mRNA abundance, e.g. ~1000 and 400 times higher than mRNA level of housekeeping genes Ppia and Ubc respectively. Apart from TRH and IGFBP2 proteins – which were confidently detected in 4/4 neonatal β cell isolates and in none of the 10-week-old β cell isolates – none of these potential biomarkers were detected by LC-MS/MS.

Neonatal islets contain a mixed population of β cells, discerned by their expression of NPY, MAFB, or DLK1

To correlate functional state of individual cells with their degree of cellular maturation, we set up immune stainings against proteins that showed intense postnatal down-regulation of their mRNA in insulin-positive cells. Some candidates – particularly those with also very low abundant mRNA such as GAP43 and EGLN3 – could not be confidently immunodetected in neonatal β cells (data not shown); others showed a degree of β cell-selectivity but without apparent postnatal downregulation (TRH, NNAT, data not shown).

We were able to confirm a clear postnatal downregulation of three markers in β cells, using independently validated in situ methods: NPY, MAFB, and DLK1 (Fig. 5). NPY expression was visualized in transgenic Npy promoter-GFP reporter mice and showed a different pattern, with distinct GFP+ and GFP− β cell subsets. A similar discerning pattern was observed for DLK1 and MAFB, using well-validated antibodies (Carlsson et al. 1997, Artner et al. 2006). All three markers disclosed heterogeneity in the neonatal β cell population: 63±15, 54±20, and 24±15% of neonatal insulin-positive cells expressed Npy-GFP, DLK1, and MAFB respectively (n=3 organs, >5000 ins+ cells counted/organ). In 10-week-old pancreas, NPY protein (not shown) and Npy-GFP could be detected in 6±3% of insulin-positive cells. At 10 weeks, the percentage of insulin-expressing cells that also stained for DLK1 (3±2%) or MAFB (1%) was considered below the limit of quantification of this method.
Basal protein synthetic activation is not a hallmark of developmental immaturity as marked by DLK1 expression

In neonates, the fraction of DLK1-expressing cells roughly corresponded to the fraction of β cells with basal hyperactivity. As DLK1 was also the most intensely regulated gene, and its apparent abundance allowed confident immunodetection, DLK1 was further used as surrogate biomarker to investigate whether the protein synthetic hyperactivity at low glucose could be directly correlated with developmental immaturity as marked by intense DLK1 expression. This was studied at the level of individual cells, in short-term (30 min) incubations of single neonatal β cell cultures with 3H-tyrosine at different glucose concentrations (Fig. 6A and B). Cells were then stained for insulin and DLK1, and after segmentation for

Figure 4
Gene clusters upregulated in neonatal β cells. Freshly isolated neonatal (red, n = 3) and 10-week-old rat β cells (blue, n = 5) were compared on Affymetrix RG230.20 oligonucleotide array. The set of 443 transcripts was at least twofold (LCB, P < 0.05) upregulated in neonatal β cells showed statistical (P < 0.001) overrepresentation of gene clusters with role in cell growth, DNA synthesis, cell cycling, and secreted factors. A selection of their most intensely regulated genes is shown from left to right: heat map indicating low (blue) to high (red) mRNA expression levels in 10-week-old adult and neonatal (neo) β cells, gene symbol and full name, NCBI accession number, fold change in neonatal/10-week-old β cells according to the microarray (array, mean of n = 3–5), and finally unpaired P value of the microarray results.
insulin positivity, β cells with high and low DLK1 fluorescence were compared for their number of labeled secretory granules per cell recorded by autoradiography (outlined in Fig. 6A). Glucose concentration dependently recruited more β cells into active protein synthesis. Yet, the protein synthetic activity, both basal and at 10 mM glucose, was indistinguishable between DLK1HIGH and DLK1LOW β cells (Fig. 6B).

Discussion

It has been known for decades that fetal and neonatal β cells, both in rodents and humans, secrete less insulin when stimulated with glucose (Asplund et al. 1969, Grill et al. 1981, Hellerstrom & Swenne 1991). This glucose unresponsiveness was attributed to an impartial differentiation, in particular relating to specialized features of glucose-sensing β cells such as NADH shuttles (Tan et al. 2002), K+ATP channels (Rorsman et al. 1989) or general immaturity with expression of disallowed genes (Aye et al. 2010, Jermendy et al. 2011). Indeed, also our neonatal β cell isolates showed a consistent flattening of glucose concentration–response curves at all tested levels: metabolic rate (NAD(P)H), insulin synthesis, and insulin secretion. We discerned two distinct features to explain this blunted responsiveness: lower activity at high glucose and higher activity at low glucose. The former is in agreement with previous studies: when a β cell matures, its maximal insulin synthetic and secretory capacity under high glucose stimulation increases.

One explanation might be that insulin-productive capacity increases with age due to cellular growth: with their 50% higher cytoplasmic volume, 10-week-old β cells theoretically can accommodate more organelles required for hormone production and release.

Another possible explanation is that neonatal β cells show a general lack of endocrine differentiation affecting all core β cell functions, e.g. proinsulin translation, folding and processing, vesicle constituents, GTPases involved in vesicle exocytosis, and incretin signaling. Our data argue against this: in fact, when specifically analyzed by oligonucleotide array for their mRNA expression of a large panel of conserved β cell marker genes (Martens et al. 2011), neonatal β cells appeared almost indiscernible from 10-week-old β cells and most statistical differences out to be noise and not confirmed by real-time PCR. Quantitative PCR using hydrolysis probes even suggested up to tenfold higher Ppia-normalized mRNA levels of classical β cell marker genes: glucokinase, GLP1 receptor, PDX1, insulin 2, IA-2/PTPRN, and HADH were just a few examples. Proteome analysis gave analogous results. LC-MS/MS analysis of unfractionated proteomes is biased toward the more abundant cellular proteins and failed to detect key regulators of β cell function/phenotype such as glucokinase, incretin receptors, or transcription factors PDX1 and NKX6.1. On the other hand, alternate-scanning LC-MS/MS has the advantage of precise and accurate quantification of relative molar abundances of the identified proteins (Silva et al. 2006, Martens et al. 2010). This showed that neonatal and 10-week-old β cells showed no major differences at the level of mitochondrial metabolism (TCA cycle, β-oxidation, and ATPase) nor at the level of proteins translation, folding,
or vesicle dynamics. Again, neonatal β cells were found to express higher levels of various classical endocrine markers, such as chromogranin A, the secretogranin family, and insulin-processing enzymes prohormone convertase 1 (PCSK1) and CPE.

The most striking difference was the concerted twofold upregulation of most glycolytic enzymes in 10-week-old β cells and the strong upregulation of the cytoplasmic enzymes (MDH1, GOT1) involved in the malate/aspartate NADH shuttle that is crucial for GSIS (Eto et al. 1999). MDH1 was four- and sevenfold upregulated at protein and mRNA level respectively. It also fits with our NAD(P)H measurements: 10-week-old β cells showed a sigmoid rise in glucose-induced NAD(P)H in the physiologically relevant glucose range centered around the fasting set point (Martens et al. 2005) and the Km of glucokinase. Neonatal β cells lacked this glucose-induced NAD(P)H accumulation, and more generally their NAD(P)H appeared already near-maximal at 5 mM glucose, with no additional effect by NADH-generating substrates nor by inhibition of NADH consumption by complex I inhibitor rotenone.

Figure 6
β cells with high or low DLK1 protein expression are indistinguishable in their in vitro proliferation rate and basal protein synthetic hyperactivity. Freshly isolated neonatal β cells were single cell-cultured and studied in short-term (30 min) glucose exposures with 3H-Tyr for autoradiographic measurement of protein synthetic activity (A and B) in individual cells. These events were correlated with DLK1 protein expression status, as outlined in (A): after segmentation of dispersed endocrine cells for insulin expression, they were dichotomously split in a DLK1HIGH (gray bars, around 25% of all cells at day 1) and DLK1LOW (black bars, around 75% cells) subset. After 30-min 3H-Tyr chase at the indicated glucose concentration, the number of individual 3H-Tyr-labeled secretory granules per cell was counted. Frequency distributions in (B) reflect the fraction of all insulin-positive cells with the indicated number of 3H-Tyr-labeled granules per cell. Bars represent mean ± s.e. of n=4 isolates; NS, not statistically different between DLK1HIGH and DLK1LOW insulin-positive cells.
We previously compared the proteomes of 10-week-old rat β cells, dichotomously FACS sorted in a subset with high glucose-induced NAD(P)H, and a subset with a more blunted glucose–NAD(P)H response (Martens et al. 2010). Interestingly, the exact same pattern was seen as with postnatal acquisition of glucose responsiveness: the highly glucose-responsive subset had a 50% higher expression of most glycolytic enzymes, with a matched 50% higher expression of MDH1 and virtually no expression differences in the mitochondrial metabolic enzymes. Higher glycolytic/MDH1 enzyme expression led to higher rates of glucose oxidation and proportionate activation of insulin synthesis and secretion. This emphasis on MDH1/glycolytic NADH as important determinant for glucose-responsive cell function and its lower activity/expression in functionally immature β cells has been observed before, both on isolated fetal rat islets (Tan et al. 2002) as on laser capture-micro (LCM)-dissected neonatal rat islets (Jermendy et al. 2011). Methodologically, LCM has the advantage that it avoids the bias on mRNA expression by ER stress imposed during lengthy isolation procedures (Marselli et al. 2009). We confirmed this but also reported that LCM significantly increases bias by contaminating exocrine acinar cells (Martens et al. 2011); evidently, it also precludes direct alignment between gene/protein expression and cellular function, as was done in our study. Nevertheless, the agreement of both approaches with regard to the role of MDH1 and NADH shutting enforces their complementary value.

The second feature was more surprising and not previously recognized: immature, neonatal β cells showed a marked hyperactivity under basal nutrient conditions: at low glucose concentrations, the smaller neonatal β cells contained more reduced NAD(P)H and had higher rates of insulin synthesis and secretion. Analysis at the single-cell level using autoradiography indicated that the basal hyperactivity of neonatal β cells was explained by a much higher fraction of β cells that showed clear biosynthetic activation at glucose concentrations below the Km of glucokinase. These findings are in line with an overall left-shift of the concentration–response curve of glucose-regulated insulin production in neonatal β cells. In fact, this left shift was previously reported by Boschero et al. who observed that neonatal β cells had higher flux through glycolysis and pentose-5-phosphate pathway at low glucose concentrations, but lower maximal levels at high glucose concentrations, in agreement with our observed insulin synthetic rates (Boschero et al. 1988, 1990).

This basal hyperactivity was reproducibly observed in >100 independent neonatal β cell isolations over 10 years in our laboratory, and it persisted for 24 h or longer after cell isolation and it was unlikely a consequence of differences in isolation technique between neonatal and adult β cells: in a separate series of experiments (Supplementary Figure 1), islets were isolated from 5-, 10-, and 40-week-old rats using the same gradient centrifugation technique as used for neonatal β cells, and here again basal hyperactivity was only seen in β cells from neonatal, and to a lesser extent 5-week-old rats.

To investigate whether this basal hyperactivity was a direct reflection of the β cells’ residual developmental immaturity at the neonatal stage, we needed protein biomarkers to differentiate less and more differentiated β cells in situ. Genome-wide mRNA profiling provided several such candidates: in line with their reported higher proliferation rate, we found a three- to fivefold higher mRNA level of gene clusters involved in DNA synthesis, cell cycling, cell growth, or tissue remodeling. Real-time PCR confirmed the activated transcription of Npy, Mafb, and Dlk1 genes in neonatal β cells, which fits with the previously observed abundant expression of NPY (Myrsen-Axcrona et al. 1997), MAFB (Nishimura et al. 2006, Arttner et al. 2007), and DLK1 (Tornehave et al. 1996) proteins in fetal and neonatal β cells. Neonatal β cell isolates contain threefold more α cells, which can introduce bias in the observed gene expression profiles. In case of NPY, MAFB, and DLK1, in situ immunofluorescence clearly indicated their expression in a subset of neonatal β cells and their downregulation at 10 weeks. The most regulated gene/protein was DLK1 (alias Pref-1, giving rise to soluble DLK1 alias fetal antigen-1 (FA1)). Also at the protein level, DLK1 was massively downregulated with age: the neonatal β cell population was clearly very heterogeneous, with ±50% of insulin-positive cells expressing high levels of DLK1 proteins and others no detectable level; when 10-week-old β cells were visualized using the same background fluorescence thresholds, virtually no positive cells could be discerned. This observation does not refute the previously observed residual expression of DLK1 in adult β cells (Carlsson et al. 1997, Friedrichsen et al. 2003), or its re-expression under stimulation with growth factors. It does underline the notion that the protein becomes significantly down-regulated with advanced maturation, as it does in so many other tissues (Jensen et al. 1994, Tornehave et al. 1996, Floridon et al. 2000, Yevtodiyenko & Schmidt 2006). Its use as in situ marker to identify the more...
immature phenotypes in a mixed cell population \textit{in vitro} thus seems valid. We therefore used DLK1 as biomarker, to verify whether the unique functional feature of the neonatal β cell, its basal hyperactivity, was a direct reflection of its (residual) developmental immaturity. The answer was unambiguous: it was not. β Cells with abundant DLK1 were equally biosynthetically (hyper-)active as those with virtually no DLK1 expression. Of note, DLK1\textsuperscript{HIGH} and DLK1\textsuperscript{LOW} β cells also did not differ in their degree of proliferation (BrdU incorporation) as previously reported (Friedrichsen \textit{et al.} 2003) and confirmed by us using EdU as label (not shown). This indicates either that DLK1 is not a good biomarker for immaturity or that the observed basal hyperactivity is not an intrinsic property of the immature β cell.

The latter option thus suggests that the basal hyperactivity is caused by extrinsic factors: it could thus be a consequence of the nutritional and hormonal extracellular environment and associated metabolic demands imposed on the β cell in the neonatal stage. Further research is needed to pin down the responsible triggers, but there are several candidates: neonatal plasma has a different amino acid profile, containing threefold more branched chain amino acids (Martens G, 2011, unpublished observations; \( P < 0.05 \)), and leucine is a potent secretagogue as well as growth factor for adult and even more for neonatal β cells (Boscherio \textit{et al.} 1990). Other possible determinants are the perinatal cortisol surge (Mastorakos \& Ilias 2003), the changes in maternal insulin resistance at the end of pregnancy (Ismail-Beigi \textit{et al.} 2006), and the high fat feeding by mother milk (Brelje \textit{et al.} 2008). Our observations not only raise the question that (combination of) factor(s) induce the basal hyperactivity but also whether this functional state might be a key trigger to drive the β cell toward proliferation. In this context, a parallel exists with maternal β cells in the second trimester of pregnancy that undergo a phenotypic shift, characterized by a left-shift of their glucose responsiveness, insulin synthetic, and secretory hyperactivity, and subsequently also associated with increased β cell proliferation (Green \& Taylor 1972, Parsons \textit{et al.} 1992, Weinhaus \textit{et al.} 1996). This also fits with the prior observations that activated maternal β cells are overall well differentiated as secretory cells also did not differ in their degree of proliferation (BrdU incorporation) as previously reported (Friedrichsen \textit{et al.} 2003) and confirmed by us using EdU as label (not shown). This indicates either that DLK1 is not a good biomarker for immaturity or that the observed basal hyperactivity is not an intrinsic property of the immature β cell.

In conclusion, this study compares gene expression and glucose-regulated functions of neonatal and 10-week-old rat β cells. It indicates that neonatal rat β cells are overall well differentiated as secretory-competent endocrine cells but require further metabolic fine tuning to become as glucose responsive as the adult β cell, both metabolically as in terms of insulin production: this involves on the one hand increasing the glucose-inducible maximal activity – likely associated with maturation of NADH shuttles leading to acquisition of a steeper glucose–NADH response – and on the other hand a down-tuning of basal activity – through mechanisms that still need to be defined. Another clear indication of the residual developmental immaturity of the neonatal rat β cells is their expression of many cell cycle- or embryonic development-related proteins such as DLK1 and MAFB. The cardinal functional feature of the neonatal rat β cells is their basal insulin synthetic and secretory hyperactivity. Using DLK1 as surrogate marker for immaturity, this hyperactivity does not appear to be a direct manifestation of their immaturity, suggesting that it is triggered by factors in the neonatal homeostatic environment.
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