Phenotypic and global gene expression profile changes between low passage and high passage MIN-6 cells

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Abstract

The long-term potential to routinely use replacement β cells/islets as cell therapy for type 1 diabetes relies on our ability to culture such cells/islets, *in vitro*, while maintaining their functional status. Previous β cell studies, by ourselves and other researchers, have indicated that the glucose-stimulated insulin secretion (GSIS) phenotype is relatively unstable, in long-term culture. This study aimed to investigate phenotypic and gene expression changes associated with this loss of GSIS, using the MIN-6 cell line as model. Phenotypic differences between MIN-6(L, low passage) and MIN-6(H, high passage) were determined by ELISA (assessing GSIS and cellular (pro)insulin content), proliferation assays, phase contrast light microscopy and analysis of alkaline phosphatase expression. Differential mRNA expression was investigated using microarray, bioinformatics and real-time PCR technologies. Long-term culture was found to be associated with many phenotypic changes, including changes in growth rate and cellular morphology, as well as loss of GSIS. Microarray analyses indicate expression of many mRNAs, including many involved in regulated secretion, adhesion and proliferation, to be significantly affected by passaging/ long-term culture. Loss/reduced levels, in high passage cells, of certain transcripts associated with the mature β cell, together with increased levels of neuron/glia-associated mRNAs, suggest that, with time in culture, MIN-6 cells may revert to an early (possibly multi-potential), poorly differentiated, 'precursor-like' cell type. This observation is supported by increased expression of the stem cell marker, alkaline phosphatase.

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Introduction

Cell replacement therapies are potential alternatives to the insulin injections presently employed to control blood glucose in type 1 diabetes. Indeed, it has been demonstrated that this condition may be cured (at least temporarily) by transplantation of pancreatic islets isolated from donor pancreata (Ricordi & Strom 2004). A major limitation, along with issues relating to immune rejection and immunosuppression, of this form of therapy is the relatively low numbers of donated pancreata compared with the increasing numbers of people developing diabetes. Alternative sources of β cells have yet to be identified (Scharfmann 2003). It has been proposed that replacement β cells may be generated from stem/progenitor cells; however, so far, an adequate supply of such cells has not been identified. Another alternative source includes the use of cultured β cell lines isolated from rodent islets (Suzuki *et al.* 2003). Although these cells have proven to be of great importance in biochemical and molecular research, a significant problem with the clinical application of these cells is the relative instability of the glucose-stimulated insulin secretion (GSIS) phenotype in long-term culture. With the increased worldwide incidence of type 1 diabetes (Gale 2005), this is an important issue to overcome and, in order to do so, a better understanding of the changes in β cell biology occurring during continuous culture is necessary if future diabetes treatments are to depend on long-term maintenance of cultured replacement tissue.

A number of β cell lines are currently available, including RIN-38, HIT-T15, INS-1, BTC6, BTC and HC9 (Santerre *et al.* 1981, Asfari *et al.* 1992, Nagamatsu & Steiner 1992, Liang *et al.* 1996), in addition to clonal populations of β cells, including INS-1 832/13, 832/1, 832/2, 832/7, 832/23, 832/3, 832/21 and 832/24, that have been engineered by transfecting with human proinsulin cDNA (Hohmeier *et al.* 2000). MIN-6 cells, originally isolated from insulinomas generated within SV40 T antigen-expressing mice and shown to share many characteristics with freshly isolated β cells (Miyazaki *et al.* 1990), have been described as a rare example of a transformed mouse β cell line that has retained many

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relevant aspects of a β cell, including GSIS (Lilla *et al.* 2003). MIN-6 cells have been characterised as being stable for 30–40 passages, i.e. with an intact GSIS response (Miyazaki *et al.* 1990). Other researchers, however, have noted only moderate GSIS (approximately 1·2-fold) responses from MIN-6 cells by passage 16 (Kayo *et al.* 1996).

In a study of low/early passage (passages 17–19) MIN-6 cells, Webb *et al.* (2000) investigated expression profiles of β cells exposed to high (25 mmol/l; continuously) and low (5·5 mmol/l; for 24 h) glucose to identify genes involved in insulin secretion (using the Affymetrix Mu6500 oligonucleotide array). The two largest clusters of genes differentially expressed between low and high glucose conditions were those encoding secretory pathway components and enzymes of intermediary metabolism. It must be considered, however, that optimised conditions for culturing β cells are often supraphysiological, i.e. HIT–T15, 10 mmol/l (Santerre *et al.* 1981); INS-1, 11·1 mmol/l (Asfari *et al.* 1992); MIN-6, 25 mmol/l (Miyazaki *et al.* 1990, Lilla *et al.* 2003, O'Driscoll *et al.* 2004); and isolated murine islets, 25 mmol/l (Zhang *et al.* 2003), although it should be noted that many researchers culture murine islets in medium containing 5·6 or 11 mM glucose. Following isolation of glucose-responsive (m9) and a non-responsive (m14) MIN-6 clonal populations at passage 18, Minami *et al.* (2000) reported ten genes (from 216 combinations of anchored and arbitrary primers) to be differentially expressed, using differential display techniques. These included glucokinase (involved in glucose phosphorylation; levels in m9>m14), stanniocalcin/STC (involved in calcium regulation; levels in m9>m14) and dlk/Pref-1 (involved in β cell differentiation and growth; levels in m9>m14). Other gene transcripts, including glucose transporter (GLUT)-2, were unchanged. Two clonal populations of MIN-6 (B1: responsive to glucose and other secretagogues; C3: non-responsive) were isolated by Lilla *et al.* (2003), and differentially expressed genes were identified using suppression subtractive hybridisation techniques and oligonucleotide (Affymetrix, MG-U74) microarrays. Genes clustered as being involved in metabolism, intracellular signalling, cytoarchitecture and adhesion were identified as being of potential interest. MIN-6-B1 cells, however, also tend to lose their GSIS beyond approximately 30 passages.

As indicated, previous studies have investigated changes in gene expression in β cells cultured in optimum glucose concentrations compared with cells grown in much lower glucose levels. Other studies have analysed selected clones of the heterogeneous β cell population. Previous studies, however, have not investigated the effects of long-term culture of these cells in medium containing glucose levels required for their optimal growth. This study aimed to investigate phenotypic and gene expression changes occurring during continuous culture of MIN-6 cells while maintaining conditions in which β cell lines and islets are generally cultured, with the long-term objective of enabling future development of potential methods whereby relevant β cell functions could be preserved in long-term culture.

Materials and Methods

Cell culture

MIN-6 cells (generously donated by Dr Yamamoto, Kumamoto University School of Medicine, Japan) were routinely maintained in Dulbecco's modified Eagle's medium containing 25 mmol/l glucose, supplemented with 20% heat-inactivated fetal calf serum and were cultured at 37 °C with 5% CO_2. Cells were re-fed every 3–4 days. Routine sterility checks, including screening for *Mycoplasma*, indicated that the cells were clear of contamination. Specific methods regarding the subculture and maintenance of MIN-6 have been described previously (O'Driscoll *et al.* 2004). All analysis presented in this study was performed on MIN-6 at passage 18, i.e. MIN-6(L/low passage) and MIN-6 at passage 40 MIN-6(H/high passage).

Proliferation assays

The proliferation rates (doubling times) of the MIN-6(L) and MIN-6(H) were determined by monitoring their growth over consecutive 24 h time periods. For this, the cells were seeded at 5×10^4 cells/well in 24-well plates (Costar, High Wycombe, UK). Cells were incubated overnight at 37 °C/5% CO_2 and three wells were trypsinised and counted on each of the 7 days following seeding. Cell doubling times were calculated from a graph of cell numbers against time.

GSIS analysis

The GSIS profiles of MIN-6(L) and MIN-6(H) were examined at glucose concentrations of 0, 3·3, 10, 16·7 and 26·7 mmol/l using methods that we described previously (O'Driscoll *et al.* 2004). To achieve this, MIN-6(L) and MIN-6(H) cells were seeded at 2×10^5 cells/well in a 24-well plate and were allowed to grow for 72 h prior to the GSIS assay. Following this, 1×KRB (Krebs–Ringer Bicarbonate) was prepared from an aliquot of frozen 10×stock (36·525 g NaCl, 2·2 g KCl, 0·941 g $CaCl_2{\cdot}2H_2O$, 1·22 g $MgCl_2{\cdot}6H_2O$ and 29·8 g HEPES dissolved in 500 ml H_2O), BSA was added to a final concentration of 0·1%, and the KRB–BSA was pH-adjusted to 7·4 with 1 mol/l NaOH. This solution was incubated for 30 min at 37 °C and 5% CO_2. Glucose concentrations of 0, 3·3, 10, 16·7 and 26·7 mmol/l were prepared in the conditioned 1×KRB and were subsequently placed at 37 °C and 5% CO_2 for 30 min. MIN-6 cells to be analysed were rinsed (twice) in 1×KRB and were equilibrated at 3·3 mmol/l glucose for 30 min at 37 °C. After equilibration, the glucose-containing stimulation media were added (1 ml/well), incubated at 37 °C and 5% CO_2 for 60 min. The GSIS assay was then terminated by placing the plate on ice. Conditioned medium (500 μl) was removed from each well, placed in an ice-cold Eppendorf tube, centrifuged at 600 ***g*** for 5 min and 200 μl supernatant were

removed for analysis by (pro)insulin ELISA (Mercodia, AB, Sylveniusgatan, Uppsala, Sweden 10-1124-10;), following the manufacturer's instructions. KCl-induced secretion was examined at 0 and 50 mM. Induction assays were performed as for glucose except that following equilibration in 1×KRB, 0 or 50 mM KCl-containing KRB were added.

Insulin content analysis

MIN-6(L) and MIN-6(H) cells (2×10^5) were re-suspended in lysis buffer (20 mmol Tris (pH 8·0), 2 mmol ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1% Triton X-100, 10% glycerol, 1·5 mmol $MgCl_2$ 137 mmol NaCl, 1 mmol, Na_3VO_4, 1X protease inhibitor cocktail (Boehringer Mannheim) and 5% BSA at 4 °C. (Pro)insulin content was assessed by ELISA (as mentioned earlier).

RNA extraction and labelling for oligonucleotide microarray hybridisation

Microarray and real-time PCR (qPCR) were performed on RNA isolated from MIN-6(L) and MIN-6(H), in three biological repeat experiments (i.e. RNA was isolated from three independent stocks of these cells, each of which resulted in a set of data). Total RNA was isolated from MIN-6(L) and MIN-6(H) using RNeasy kit (Qiagen), following the manufacturer's instructions. RNA quantity and purity were assessed spectrophotometrically (Nanodrop ND-1000, Labtech International, Ringmer, East Sussex, UK). Gel electrophoresis and Agilent Bioanalyser were used to assess RNA qualitatively after isolation, after biotin-labelling and post-fragmentation. Double-stranded cDNA was synthesised from 10 μg total RNA using SuperScript II RT (Invitrogen Life Technologies) and T7-Oligo(dT)24 promoter primer (Affymetrix, Mercury Park, High Wycombe, UK) to initiate reverse transcription of mRNA in the specimens. Following clean-up of double-stranded cDNA using the GeneChip Sample Cleanup Module (Affymetrix), biotin-labelled cRNA was synthesised using the Enzo Bioarray HighYield RNA Transcription Labelling Kit (Affymetrix), purified using IVT cRNA Cleanup Columns (Affymetrix) and fragmented to products of 35–200 bases.

Microarray hybridisation

Hybridisation solution (1 mol/l NaCl, 20 mmol/l EDTA, 100 mmol/l 2-(*N*-morpholino)ethanesulphonic acid and 0·01% Tween 20) was used to pre-hybridise test arrays (test 3: Affymetrix) and, subsequently, Affymetrix MG-U74 oligonucleotide microarrays for 10 min at 45 °C. The pre-hybridisation solution was removed and replaced with 200 μl hybridisation solution containing 0·05 μg/μl fragmented cRNA. The arrays were hybridised for 16 h at 45 °C. Arrays were, subsequently, washed (Affymetrix Fluidics Station 400) and stained with streptavidin–phycoerythrin (Stain Buffer, 2 mg/ml acetylated BSA and 10 μg/ml streptavidin R–phycoerythrin; Molecular Probes, Inc., Eugene, OR, USA), and were scanned on a 2500 GeneArray scanner. Resulting data were analysed using Affymetrix Microarray Suite 5.1, Affymetrix Data Mining Tool 2.0, GeneSpring (Agilent Technologies, Lakeside, Stockport, Cheshire, UK), Bibliosphere (Genomatix, Bayerstr, München, Germany) and PathwayAssist (Ariadne Genomics, Rockville, MD, USA).

Bioinformatics criteria for selecting induced/suppressed genes and functional assignment

All the data from the biological triplicates assessed were normalised using the model-based probe logarithmic intensity error algorithm (see 'Guide to Probe Logarithmic Intensity Error', Affymetrix tech note). Following data normalisation, gene lists were generated using a *t*-test to identify genes that were significantly ($P<0·01$) differentially expressed between the three MIN-6(H) arrays and the MIN-6(L) arrays (used as the reference/'control'). These gene lists were segregated into those containing genes that were either significantly higher, or lower, in the MIN-6(H) cells compared with the MIN-6(L) cells. A two-fold cut-off (increase or decrease) was then applied.

Real-time PCR (qPCR)

cDNA was synthesised from 1 μg total RNA, using MMLV-RT (Sigma), in a 20 μl reaction volume (O'Driscoll *et al.* 1993). The cDNA was amplified, by qPCR, using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). For this purpose, relevant primers were designed (see Table 1) and double stranded (ds)DNA-specific dye SYBR Green I/Taqman was incorporated for quantitative detection of PCR products. The temperature profile of the reaction was 95 °C for 10 min, 30 cycles of denaturation at 95 °C for 40 s, annealing (see Table 1 for specific temperatures) for 40 s and extension at 72 °C for 40 s, followed by 72 °C for 7 min. C_T (threshold cycle) values were used in this study. An internal housekeeping gene control, β-actin (expression levels of which were found to be similar in MIN-6(L) and MIN-6(H) cells), was included to normalise differences in RNA isolation, RNA degradation and efficiencies of the qPCR. The relative quantity of expression in MIN-6(L) was set at 1; changes in fold expression in MIN-6(H) were calculated relative to levels in MIN-6(L).

Alkaline phosphatase staining and activity assay

Alkaline phosphatase staining of MIN-6(L) and MIN-6(H) was performed by adding BCIP (5-bromo-4-chloro-4-indolyl-phosphate, 4 toluidine salt (Roche)) and NBT (nitroblue tetrazolium chloride) in phosphatase buffer (100 mmol/1 Tris, 100 mmol/1 NaCl, 50 mmol/1 $MgCl_2$) to cells. BCIP acts as substrate for alkaline phosphatase and the 5-bromo-4-chloro-3-indoxyl formed results in an insoluble

Table 1 Validation of microarray data (induced/upregulated and downregulated in MIN-6(H) versus MIN-6(L)) by qPCR

	Function/physiological role	Primer sequence (5′–3′)	Microarray[a] (fold)	Real-time PCR[b] (fold)
Induced/upregulated				
Secretion				
gck	Phosphorylation of glucose to G-6-P	gatgctggatgacagagccaggatg agatgcactcagagatgtagtcga	NS	1·08–1·26
GLUT-2	Facilitative glucose transporter	Taqman	NS	1·73–7·14
Adhesion				
tjp	Paracellular permeability barrier	acgacaaaacgctctacagg gagaatggactggcttagca	2·2	1·32–2·14
Differentiation				
Nestin[c]	Marker of neuronal-like cells	ggctacatacaggattctgctgg caggaaagccaagagaagcct	4·2	1·28–2·39
dll1	Controls development of neurons	agccctccatacagactctc cagaacacagagcaaccttc	5·13	1·28–1·63
trp53	Has been found to negatively impact terminal differentiation	agctttgaggttcgtgtttg ggaacatctcgaagcgttta	2·1	1·17–2·05
Downregulated				
Secretion				
egr1	Elicited by glucose secretagogues	atggacaactaccccaaact attcagagcgatgtcagaaa	5·54	5–9·8
pld1	Regulates GSIS	tcttatcccttcctgctacc ccaccttgttgaacacaact	4·07	7·2–27
scg3	Associated with secretory granule membrane	ggatactggtgttggtgctc tttctgttattgccctcagc	3·45	4·5–6
chgb	Islet secretory glycoprotein	atccaagtccagtgttccaa tctcattgcctaccttcgtc	2·96	16·4–25·6
sgne1 (7B2 protein)	Required for PC2 activation.	ttcagtgaggatcaaggcta ctgggtagtcatgttctgga	3·55	5·9–9·9
pcsk2	Processes proinsulin to mature insulin	cacagatgactggttcaaca tcacagttgcagtcatcgta	2·56	5·4–8·1
cck	Stimulates insulin secretion from β cells	gatggcagtcctagctgctg ccaggctctgcaggttctta	6·53	A
Proliferation				
dlk1	β cell autocrine/proliferation factor	Taqman probe	2·2	P
Adhesion				
CEACAM1[d]	Cell adhesion and proliferation suppressor.	aatctgcccctggcgcttggagcc aaatcgcacagtcgcctgagtacg	25·4	28·6–45·5
ODC	Inhibition may prevent polyamine-regulated β cell replication	ctgccatacctcttgatgaag cgttactggcagcatacttg	1·92	1·12–1·41
Differentiation				
Pax 6	Expression required for islet cell development; facilitates GSIS	aagagtggcgactccagaagt accatacctgtattcttgcttcagg	2·17	3·4–3·6
Pax 4	Transcription factor involved in pancreatic development	tggcttcctgtccttctgtgagg tccaagacacctgtgcggtagtag	NS	0·97–1·55
pdx1	Essential for pancreatic development and insulin secretion	ggtggagctggcagtgatgtt accgcccccactcggggtcccgc	NS	2·2–3·0
Isl1	Development of the dorsal pancreatic bud and all endocrine islet cells	acgtctgatttccctgtgtgttgg tcgatgtggtacaccttagagcgg	1·47	1–2·9
Nkx2.2	Pancreatic cell and neuron development	cggagaaaggtatggaggtgac ctgggcgttgtactgcatgtgctg	NS	1·8–3·2
Beta2/neuroD	Pancreatic, hippocampus and cerebellum development	cttggccaagaactacatctgg ggagtagggatgcaccgggaa	NS	1·9–2·3
NPY	Induced in insulin$^+$ cells at islet formation	tgaccctcgctctatctctg aagggtcttcaagccttgtt	23·98	6·7–200
gap43	Neurite outgrowth and synaptic plasticity	gctgctaaagctaccactga taggtttggcttcgtctaca	2·03	3·4–5·9
Insulin				
PPI		agcgtggcttcttctacacacc ggtgcagcactgatccaccatg	NA	2·2–2·8
INS I[e]		tagtgaccagctataatcagag acgccaaggtctgaaggtcc	NS	2·6–10·2

(continued)

Table 1 *Continued*

	Function/physiological role	Primer sequence (5′–3′)	Microarray[a] (fold)	Real-time PCR[b] (fold)
INS II[e]		ccctgctggccctgctctt aggtctgaaggtcacctgct	NS	1·9–3·2
β-Actin	β-Actin was co-amplified with other cDNAs as endogenous control, annealing temperature (temp.) used was as relevant for cDNA of interest	gaaatcgtgcgtgacattaaggagaagct tcaggaggagcaatgatcttga & Taqman		

qPCR, quantitative/real-time PCR; NA, not represented on array; NS, not significant.
[a]Mean of three microarray experiments.
[b]Range of fold changed detected by $n=3$ qPCR biological repeats (in triplicate); P, induced in MIN-6(H) from absent in MIN-6(L); A, present in MIN-6(L) but absent from MIN-6(H).
[c]All primers, except for Yu *et al.* (2002).
[d]All primers, except for Han *et al.* (2001).
[e]All primers, except for Lumelsky *et al.* (2001) and Taqman primers, were selected in our laboratory.

purple/brown stain. After allowing colour to develop, cells were washed thrice in H_2O and photographed.

For alkaline phosphatase activity analysis, MIN-6(L) and MIN-6(H) cells were seeded at 1×10^4 cells/well in 96-well plates and were cultured for 48 h. After 48 h, medium was removed, the cells were washed in PBS (pre-heated to 37 °C), followed by the addition of 100 µl reaction buffer (1 M diethanolamine (Sigma) pH 9·8 (at 37 °C) 0·5 mM $MgCl_2$ (Riedel de Haen, Wunstor Ferstrasse, Germany), 0·01 M *p*-nitrophenol phosphate (PNP; Sigma), 0·1% Triton X-100 (Sigma)). The reaction buffer was warmed to 37 °C prior to addition to the cells. A blank/control was prepared by adding water to the reaction buffer. Upon addition of the reaction buffer to the cells, the optical density $(OD)_{405}$ was measured immediately and then at 5-min intervals, over a 20-min period. The temperature of the assay plate was maintained at 37 °C. The activity of alkaline phosphatase in each case was defined as:

$$\Delta\text{OD sample} - \Delta\text{OD blank}/18{\cdot}2(\text{millimolar extinction coefficient of PNP under these assay conditions})$$

In each case, the activity was normalised to protein content. This activity assay was performed on triplicate biological samples.

Results

Phenotypic analysis of MIN-6(L) and MIN-6(H)

Continuous subculturing resulted in an increase in proliferation rate. Growth curves, plotted over 7 days (Fig. 1) following seeding indicated that the rate of proliferation in MIN-6(H) cells is approximately twofold greater than that of MIN-6(L). MIN-6(H) appeared to still be in log phase day 7 (168 h); however, MIN-6(L) apparently reached plateau phase after approximately 6 days (144 h), when the colonies reach their 'maximum' size. Furthermore, re-seeding these cells into new tissue culture flasks and culturing for approximately 1 month (re-feeding every 3–4 days) the MIN-6(L) cells remain viable, but proliferate much more slowly than the MIN-6(H) cells. The MIN-6(H) population shows a significantly greater increase in total cell number over approximately 30 days, suggesting that MIN-6(H) cells have greater proliferation potential, under the conditions used here, than MIN-6(L) cells.

Insulin content and stimulated insulin secretion

Similar to our previous analysis of MIN-6 passages 17–22 compared with passages 40–49 (O'Driscoll *et al.* 2004), analyses

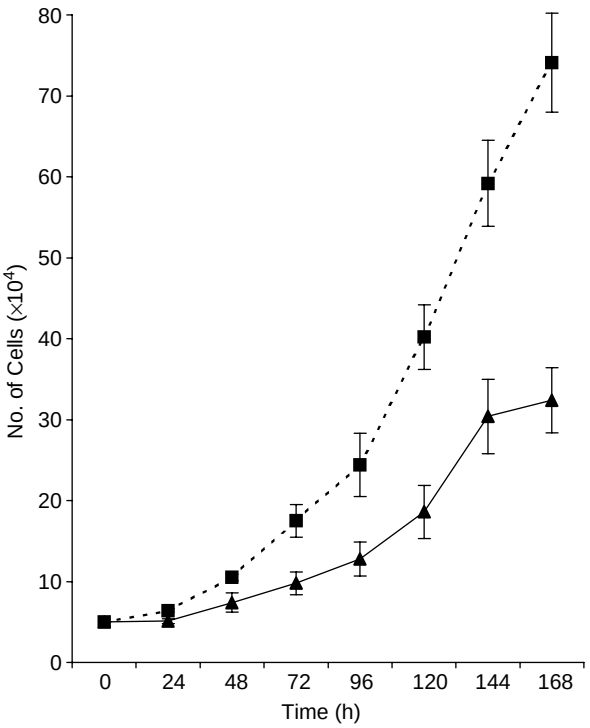

Figure 1 Proliferation data comparing MIN-6(L) (straight line) and MIN-6(H) (dotted line). Cells were cultured in 24-well plates and three wells were counted each day. The experiment was repeated on more than three independent cell populations. The data presented equals mean cell number ± S.D.

of three independent stocks of MIN-6(L) and MIN-6(H) indicate that both cell populations have similar levels of (pro)insulin content (3·73±0·34 in MIN-6(L), compared with 3·57±0·39 pmol/ml per mg protein in MIN-6(H)), although they differ in their (pro)insulin secretory properties, i.e. the ability to regulate the secretion of insulin in response to external glucose signals. Basal (pro)insulin secretion from MIN-6(H) cells was slightly, but not significantly, greater than that for MIN-6(L) in glucose-free conditions; this basal secretion rate was maintained, despite increasing environmental glucose levels (Fig. 2). MIN-6(L) cells, however, increased insulin secretion by a factor of approximately five to sixfold over the entire glucose range tested (0–26·7 mmol/l), demonstrating a functional GSIS response. Although MIN-6(H) did not exhibit a functional GSIS response, an approximately sixfold KCl-induced insulin secretion was achieved with these cells, with no significant ($P=0·6$) difference in response to KCl detected between MIN-6(H) and MIN-6(L) cells.

Effects of continuous passaging on global gene expression in MIN-6 cells

Passaging of MIN-6 cells resulted in many changes in gene expression, including both upregulation and downregulation of many transcripts. Through the careful standardisation of the culture conditions used for the MIN-6 cells, good concordance between biological repeats was obtained (Fig. 3A), increasing confidence in the final gene lists obtained. Approximately, 4% (500) of the transcripts on the microarray were significantly increased and approximately 3·7% (461) were significantly decreased in three biological repeat studies of MIN-6(H) compared with MIN-6(L). Of the 500 products upregulated, 65 were described as RIKEN cDNAs and five were expressed sequence tags (ESTs); 64 RIKEN cDNAs and eight ESTs were identified in the 461 transcripts found to be downregulated with increased passaging. Overall, however, comparison of genes flagged 'present' in MIN-6(H) compared with MIN-6(L) cells indicated that these cell lines are fundamentally different (Fig. 3B).

Eighty-eight genes were overexpressed twofold or more in MIN-6(H), while 185 were downregulated by this factor in MIN-6(H) compared with MIN-6(L). As indicated in Fig. 4, genes involved in crucial aspects of β cell biology, including metabolism, cell adhesion, growth and protein secretion, were amongst the largest groups of genes affected. For several genes of interest, including, as examples, genes involved in the categories listed above, the relative expression levels were analysed by qPCR and generally resulted in confirmatory results (Table 1).

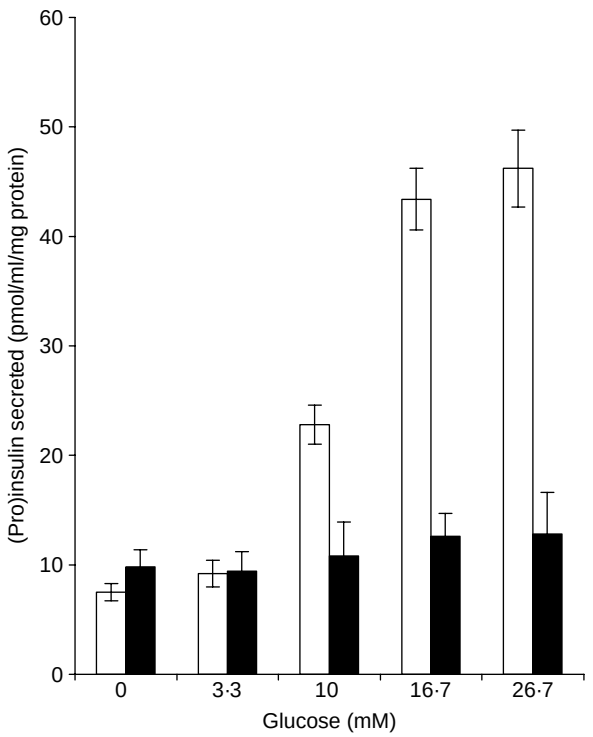

Figure 2 (Pro)insulin secretion profiles with increasing glucose concentrations in low (white bars) and high (black bars) passage MIN-6 cells. Results are presented as pmol/ml per mg protein to allow for the increased growth rate of high passage MIN-6 cells. Results represent means±S.D. from all three experiments.

Alkaline phosphatase expression and activity

Alkaline phosphatase activity is a characteristic of undifferentiated stem cells. Using BCIP as a substrate for alkaline phosphatase, Fig. 5 indicates that the extent of alkaline phosphatase staining is much more widespread in MIN-6(H) than in MIN-6(L) populations. This is consistent with the previous observations indicating that continuous culture may be resulting in de-differentiation and is supported by our finding of a 1·4 (±0·06)-fold greater alkaline phosphatase activity in MIN-6(H) cells compared with MIN-6(L) cells.

Discussion

Overall findings

In this study, almost 1000 genes were considered significantly differentially expressed ($P\leq0·01$) in high passage MIN-6(H) cells compared with MIN-6(L). Genes involved in regulated insulin secretion, proliferation, adhesion and development were among those affected. MIN-6(H) adopted less differentiated, poorly specialised, characteristics distinctly different from MIN-6(L).

Secretion

The loss of gene transcripts involved in the processing and regulated secretion of insulin with increased passage number has also been observed previously, leading to the proposal of β cell de-differentiation (Kayo *et al.* 1996). In our studies, MIN-6(H) cells no longer exhibit GSIS. Whereas our microarray analyses

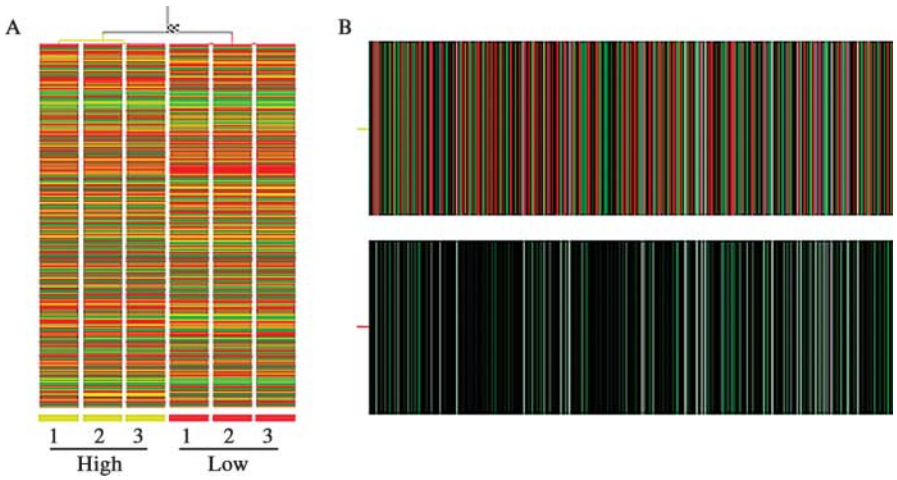

Figure 3 (A) Spearman correlation analysis of the six samples making up the 'control' (MIN-6(L)) and 'test' (MIN-6(H)) samples indicates the high quality and reproducibility of the biological replicates, as indicated by the close clustering of the test and control samples into two discrete groups illustrated in the condition tree above. Up- and downregulated genes are represented by red and green respectively. R (Spearman correlation coefficient) between triplicate MIN-6(L) is 0·991; between triplicate MIN-6(H) is 0·986 and between MIN-6(L) and (H) is 0·96. (B) Hierarchical clustering analysis of the 6605 genes flagged 'present' in this analysis (where each vertical line is a gene) clearly demonstrate the large differences in global gene expression upon serial passaging of MIN-6 cells.

indicated that mRNAs for 'glucose sensory apparatus' components GLUT-2 and GCK (as well as other hexokinase family members (I, II and III) and GLUT-1), were apparently not significantly affected by continuous passaging, levels of expression of many gene products generally associated with vesicle formation/transportation and secretion were significantly lower in MIN-6(H) compared with MIN-6(L) cells. Examples include nucleobindin (Lin *et al.* 1998), pro-PC2 (Furuta *et al.* 1998), PC2 chaperone protein 7B2 (Marzban *et al.* 2005), secretogranin V (Furukawa *et al.* 1999) and reticulon. Similarly, synaptotagmin 1, which has been shown to co-localise in β cells with synaptotagmin 7 (function of which is as a vesicular Ca^{2+} sensor for exocytosis in endocrine cells (Fukuda *et al.* 2004)), is downregulated approximately 1·7-fold in MIN-6(H), compared with MIN-6(L). Downregulation of *egr1*, *pld1*, *scg3*, *chgb*, *sgne1* and *ck* gene transcript expression (see Table 1), with increased passaging, may also contribute to this loss of GSIS. It is possible that Ass1 (results in increased Ca^{2+} levels, is implicated in insulin release via the citrulline–arginosuccinate–arginine cycle in cells (Nakata & Yada 2003)) is also a contributing factor to this phenomenon. Transcript levels of this gene were found to be upregulated in MIN-6(H). Recent evidence has suggested that syntaxin 4 is a crucial facilitator of GSIS in pancreatic β cells (Spurlin & Thurmond 2006). In this study, syntaxin 4 mRNA levels were significantly downregulated in MIN-6(H) ($P=0·01$) with a fold change of slightly less than twofold.

Adhesion/proliferation

Comparison of the insulin levels, secretion rates and GSIS capabilities of intact islets, dispersed islets and re-aggregated islets revealed that the dispersed and re-aggregated cells had impaired responses to glucose (Linzel *et al.* 1998). Thus, cell-cell attachments are crucial for correct GSIS. Upon continuous culture, it was clear that MIN-6 cells no longer exist as 'clumps' and that they tend to form more conventional monolayers. Microarray analysis indicated that the transcript levels of a number of cell adhesion molecules were greatly reduced with passaging, including CEACAM-1, CEACAM-2, contactin 1 and E-cadherin, with increased expression of tight junction protein and claudin 11. The loss of E-cadherin in non-responsive β cells is in agreement with a similar observation by Lilla *et al.* (2003) in a non glucose-responsive MIN-6 clone (C3) and its known association with undifferentiated pancreatic progenitor cells (Jensen *et al.* 2005). Furthermore, in agreement with this study, reelin (involved in cell–cell aggregation) transcript levels in MIN-6(H) were much lower than in MIN-6(L). A major component of cell–cell communication in pancreatic islets involves the connexins, particularly C×43 (Meda *et al.* 1991) and C×36 (Calabrese *et al.* 2001). These transcripts were, however, flagged absent in our studies. This may reflect a limitation of microarray studies where probe selection can exclude important transcripts from final gene lists. Although our experience has been that present calls on the microarray are generally confirmed by RT-PCR/qPCR, exceptions to this are GLUT-2 (where qPCR in one experiment did not confirm microarray results) and dlk1 (where triplicate microarrays indicated more than twofold downregulation in MIN-6(H) compared with MIN-6(L), but qPCR suggested an 'absent' to 'present' call, in this comparison). These limited conflicting results between microarrays and qPCR are likely

to reflect differing locations of microarray probes (typically within the 3′ UTR) and qPCR primers.

CEACAM-1 may have an additional role in the loss of differentiation state and GSIS in cultured MIN-6 separate from its role in adhesion, as it can act as a suppressor of cellular proliferation (Singer *et al.* 2000, Han *et al.* 2001); thus, its absence at high passages may, in fact, promote growth. Other proliferation-related genes were found to be altered in MIN-6(H), including increased levels of cdk4 mRNA expression and decreased levels of dlk1 mRNA, which respectively, promote

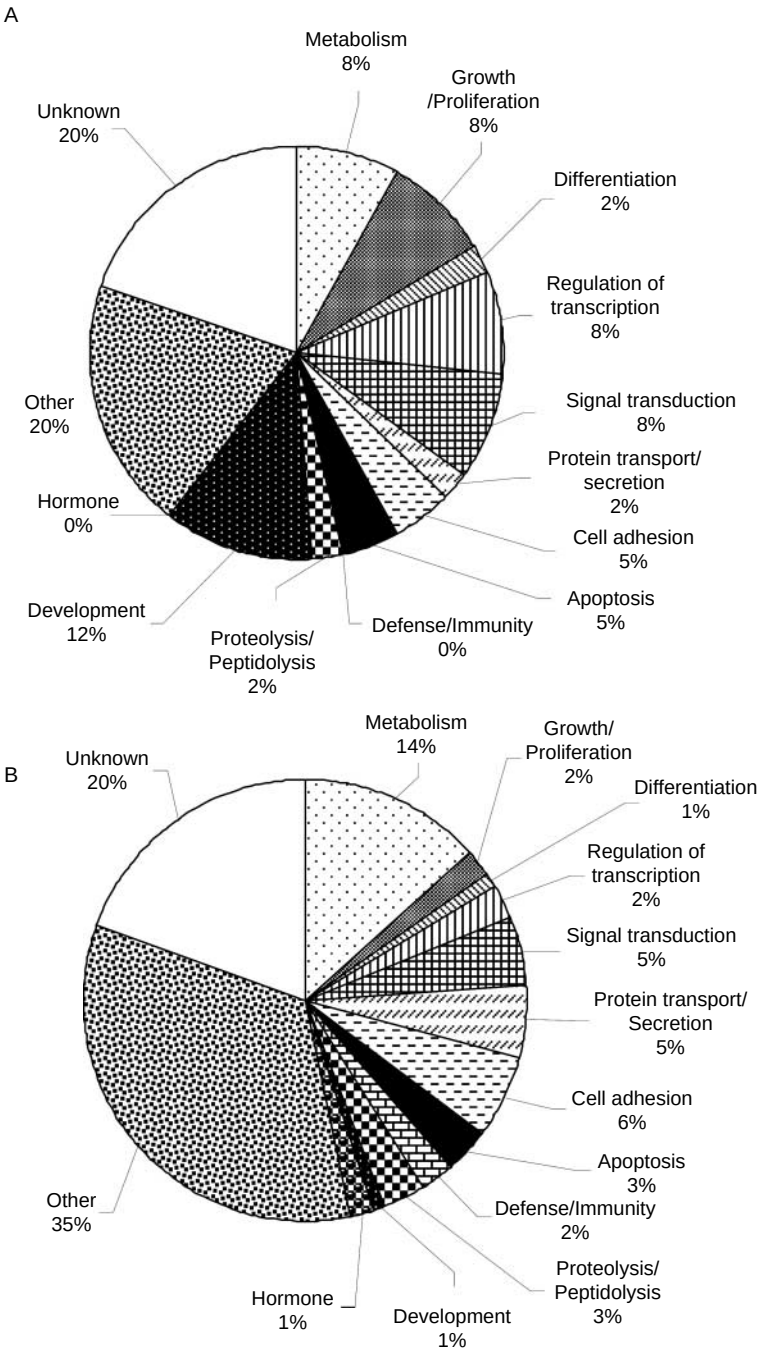

Figure 4 (*continued*)

C

Breakdown of "Other" Categories in Pie-Charts representing (A) Up- and (B) Down-Regulated Transcripts		
Category	Up (%)	Down (%)
Actin binding	1·2	0·5
Aminopeptidase	0·0	0·5
ATP binding	2·3	1·0
Calcium ion binding	1·2	4·0
Calmodulin binding	1·2	0·0
Catalytic activity	0·0	0·5
Cell-Cell signalling	0·0	0·5
Cytoskeleton	1·2	0·5
DNA binding	1·2	0·0
Electron transport	0·0	0·5
Endonuclease	0·0	1·0
Endopeptidase inhibitor	0·0	1·5
Epigenetic regulation of gene expression	0·0	0·5
GTP binding	0·0	0·5
Hydrolase activity	1·2	1·5
Ion transport	0·0	4·0
Membrane	0·0	0·5
Metal ion binding	1·2	3·5
Oxidoreductase	0·0	3·0
Protein binding	5·8	5·0
Protein folding	0·0	0·5
Protein glycosylation	0·0	0·5
Protein translation	0·0	0·5
Receptor	0·0	0·5
RNA binding	3·5	2·0
Small molecule transport	0·0	0·5
Steroid biosynthesis	0·0	0·5
Transferase	0·0	0·5

Figure 4 Representations of the overall categories of gene transcripts that were either (A) upregulated or (B) downregulated in MIN-6(H), compared with MIN-6(L), cells. The categories represented by 'other' are listed in (C). The 'unknown' category includes RIKEN cDNAs and newly discovered gene products that have not yet been assigned functions.

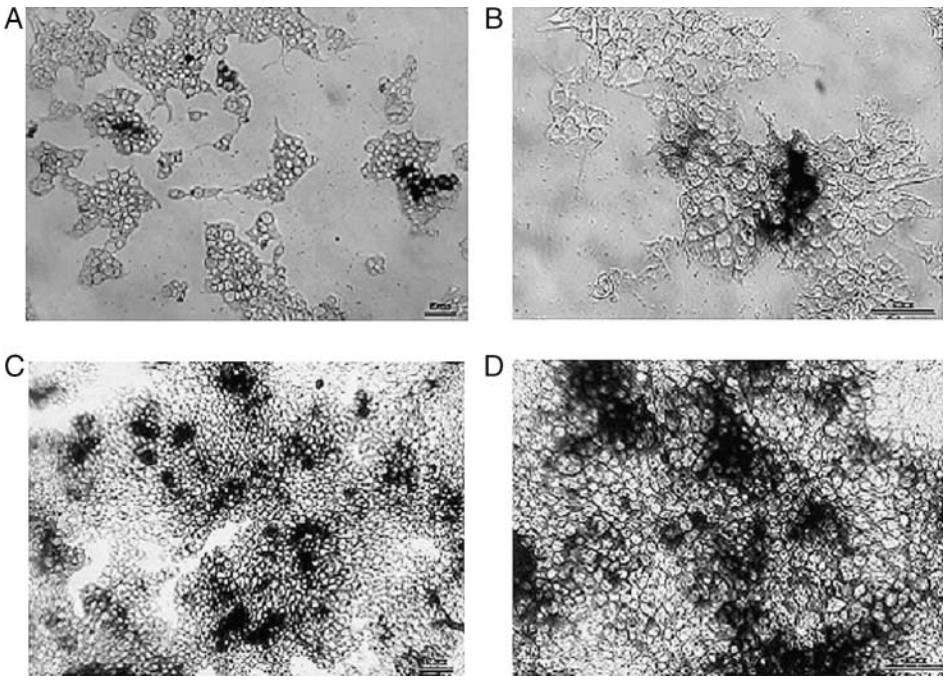

Figure 5 MIN-6 cells stained for alkaline phosphatase expression. MIN-6(L) cell population (A and B) were found to have much less staining than MIN-6(H) (C and D). Scale bar=50 μm in each case.

(Marzo *et al.* 2004) and attenuate (Friedrichsen *et al.* 2003) proliferation of β cells. This loss of dlk1 in non-responsive β cells is in agreement with the study by Minami *et al.* (2000). The increased proliferation rate of MIN-6(H) cells (also described by Sawada *et al.* (2001)) may also be associated with the impaired insulin processing and secretion. Zimmer *et al.* (1999) engineered MIN-6 cells that could be held in a quiescent state using a '*tet* off' system and elegantly demonstrated that growth arrest correlated with increased levels of secretory granule genes, chromogranin A and insulin. Similarly, in our laboratory, it has been observed that particular batches of fetal calf serum result in increased proliferation of MIN-6 cells and concomitant loss of GSIS, necessitating careful screening of new serum batches (unpublished observations).

De-differentiation and/or emergence of a poorly differentiated subpopulation

The loss of GSIS coupled with increased proliferation, the morphological changes observed and the increase in the level and activity of the stem cell marker alkaline phosphatase, are all indicative of a major transition occurring within serially passaged MIN-6 cells. These changes, particularly the loss of the inherent GSIS capability of β cells, and the increased proliferation, apparently involve functional de-differentiation of the cell population, but whether this is due to de-differentiation of the population as a whole, or overgrowth by a faster growing poorly differentiated subpopulation, remains unclear. It must be remembered that MIN-6 is a transformed cell line, not a completely normal β cell. Studies of MIN-6 clonal populations have shown that clones with a high and stable level of GSIS do eventually lose this phenotype, suggesting a model in which fast-growing poorly differentiated cells arise, perhaps by mutation; these cells may then overgrow and come to dominate the differentiated populations. This observation was previously made in a rat insulinoma cell line, INS-1, where clonal selection resulted in populations that exhibited markedly enhanced and stable insulin secretion responsiveness to glucose and its potentiators (Hohmeier *et al.* 2000). Less differentiated β cells, such as RINm5F, have higher proliferative rates than more differentiated β cells. This effect has previously been linked to the increased expression of furin in less differentiated β cells. However, furin mRNA levels were not significantly different between MIN-6(L) and MIN-6(H) cells.

The reduction in Pax 6 levels, coupled with the appearance of nestin, correlates with the less differentiated state of the high passage cells. Nestin has been used as a marker of cells that can differentiate towards a β cell-like cell type (Lumelsky *et al.* 2001). The increased mRNA levels of other neural markers, such as delta-like 1 (De Bellard *et al.* 2002, Grandbarbe *et al.* 2003), cerebellin-1 (Albertin *et al.* 2000), junctophilin type 3 (Nishi *et al.* 2002) and necdin, also tend to agree with the hypothesis that the high passage MIN-6 cells may be reverting to a more primitive, neuroendocrine-like cell type; in fact, most of the 'development'-associated genes in the two-fold upregulated gene list (Fig. 4A) are actually involved in neurogenesis and brain development.

The origins of pancreatic cells and neurons have many crucial transcription factors in common, including ngn3, Isl1, NeuroD1/Beta 2, Pax 6 and Nkx2.2 (Edlund 1999); many of which are differentially expressed in low and high (Table 1) passage MIN-6 cells. Furthermore, brain tumours have been identified that express insulin and proinsulin, resulting in severe hypoglycaemia for the patient (Nakamura *et al.* 2001). This suggests that both neuronal and β cells may have similar progenitor cells and that it is these cell types to which de-differentiating MIN-6 cells are reverting. This hypothesis is strongly supported by the recent report by Seaberg *et al.* (2004) suggesting a novel endodermal/ectodermal multi-potential precursor in embryonic development that persists in adult pancreata and also by the recent *in vitro* differentiation of rat neural stem cells into an insulin-expressing phenotype expressing functional responses typical of pancreatic β cells (Burns *et al.* 2005).

The increased expression of nestin and basp1 mRNA in parallel with decreased levels of *GAP43* gene transcript expression in MIN-6(H), compared with MIN-6(L), indicates that transforming growth factor (TGF)β1 plays at least a partial role in the de-differentiation towards a 'neuroendocrine precursor' cell type. TGFβ1 levels are almost sixfold lower in MIN-6(H) cells, and under normal circumstances, would have a negative effect (Loo *et al.* 1995) on nestin levels (upregulated in MIN-6(H)) and a positive effect on GAP43 levels (down-regulated in MIN-6(H)). While basp1 and GAP43 can functionally substitute for one another, GAP43 negatively affects basp1 expression (Frey *et al.* 2000), which may explain why basp1 levels are threefold upregulated in MIN-6(H). As TGFβ1 positively regulates clusterin levels (Jin & Howe 1997), the reduction in TGFβ1 may also explain the unexpected decrease in clusterin levels, which would possibly have been expected to be elevated in more rapidly proliferating MIN-6(H) cells (Kim *et al.* 2001). The reduced levels of TGFβ1 are also in keeping with the more rapidly proliferating phenotype of MIN-6(H) as TGFβ1 is a potent growth inhibitor.

This study describes differences between glucose responsive, low passage MIN-6 cells and non-glucose responsive, MIN-6(H) cells. Our overall observation is that the two cell types, while still having many attributes in common, are essentially very different. MIN-6(H) have a less differentiated phenotype, upon serial passage in culture, and this is associated with a higher proliferation rate. The questions that must now be addressed are functional in nature, e.g. can this change be prevented? Several genes that are involved in crucial β cell functions have been identified as being potentially no longer operational in MIN-6(H). Some of these are linked to proliferation, e.g. dlk1. These genes are targets for functional studies to identify whether de-differentiation can be prevented by controlling proliferation, e.g. by re-expression of these products in high passage β cells. Identification of procedures to continuously culture β cell models such as MIN-6 cells, without the loss of GSIS and other

β cell characteristics, may be important in the generation of therapeutic cells for replacement therapy of type 1 diabetes.

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