Genes induced by growth arrest in a pancreatic β cell line: identification by analysis of cDNA arrays

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Received 16 June 1999; received in revised form 19 July 1999

Abstract Pancreatic β cell lines are a potentially attractive source of material for cell therapy of insulin-dependent diabetes mellitus. However, induction of proliferation in post-mitotic, differentiated β cells is likely to affect the expression of multiple genes associated with cell function, resulting in dedifferentiation. We have developed a murine β cell line by conditional transformation with the SV40 T antigen oncoprotein. These cells can undergo reversible induction of proliferation and growth arrest. Here we utilized this model to identify differences in gene expression between proliferating and quiescent β cells, by analyzing known β cell genes and differentially secreted proteins, as well as by a systematic survey of a mouse cDNA array. Our findings demonstrate that growth arrest stimulates expression of the insulin gene and genes encoding components of the insulin secretory vesicles. Screening of the cDNA array revealed the activation of multiple genes following growth arrest, many of them novel genes which may be related to β cell function. Characterization of these genes is likely to contribute to our understanding of β cell function and the ability to employ β cell lines in cell therapy of diabetes.

Key words: Beta cell gene expression; Insulin biosynthesis; Beta cell line; cDNA array; Conditional transformation

1. Introduction

Insulin biosynthesis in pancreatic β cells and its regulated secretion in response to physiological signals depend on expression of a set of genes, which together define the differentiated state of these cells. Our understanding of these functions relies on identification of the gene products involved in them and characterization of their interactions. It is conceivable that only a fraction of the genes involved in β cell function are known at present. The development in recent years of improved techniques for identification of differentially expressed genes has accelerated the rate of discovery of new genes expressed in differentiated β cells. Comparisons between genes expressed in mouse β and α cell lines [1–3], and between human pancreatic islet and acinar tissue [4], have identified numerous novel genes, as well as known genes not previously reported to be expressed in β cells. Similarly, genes induced in β cells by glucose were identified by comparing transcripts present at high glucose with those at low glucose in rat islets [5] and mouse insulinoma cell lines [6,7]. The differential display technique has been used to identify genes turned on during hamster islet neogenesis [8], and during differentiation of rat AR42J cells into insulin-producing cells following treatment with activin A and betacellulin [9]. These approaches are limited to the changes in gene expression that are reflected at the transcriptional level, and overlook changes due to translational effects or post-translational protein modifications. Nevertheless, these studies have begun to unravel the complexity of the gene expression configuration of differentiated β cells.

Since the amount of RNA needed for analysis of differential gene expression is relatively large, many of these studies have utilized transformed cell lines rather than primary islets. While β cell lines provide an abundant source of pure β cells, their pattern of gene expression may differ substantially from that of normal β cells. Under normal conditions, differentiated β cells do not replicate, and probably must withdraw from the cell cycle to fully express their specialized properties. Beta cell replication and β cell function are likely to involve the expression of mutually exclusive sets of genes, as evidenced by the poor differentiation of fetal islets and many β cell lines. We developed a murine β cell line, βTC-tet, conditionally transformed by the SV40 T antigen (Tag) oncoprotein under control of the regulatory elements of the bacterial tetracycline (tet) operon [10]. Proliferation of these cells depends on Tag, and they undergo growth arrest in its absence. Growth arrest results in enhanced cell differentiation, as evidenced by an elevated insulin content [11], and by downregulation of genes abnormally turned on in the proliferating βTC-tet cells, such as hexokinase [11] and preproglucagon [12]. Here we describe a systematic survey of changes in gene expression that occur during differentiation of βTC-tet cells following growth arrest, with emphasis on those genes that are turned on by this process and may be related to β cell function.

2. Materials and methods

2.1. Cell culture

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and supplemented with 15% horse serum, 2.5% fetal calf serum, and penicillin/streptomycin (all cell culture products were from Biological Industries, Israel). Growth arrest was induced by addition of tetracycline (Sigma) to a final concentration of 1 μg/ml for a period of 10–14 days. The experiments described here were performed with cells in passages 14–27.

2.2. RNA preparation and Northern blotting

Poly(A)$^+$ RNA was prepared with an mRNA isolation kit (Boehringer Mannheim). One μg of poly(A)$^+$ RNA was loaded on 0.8% agarose gels containing 40 mM triethanolamine, 2 mM EDTA and 2 M formaldehyde, and electrophoresed in running buffer containing 40 mM triethanolamine and 1 M formaldehyde. The RNA was blotted onto nylon membranes (Boehringer Mannheim) and crosslinked to the membrane by UV. Membranes were stained in 0.5 M sodium acetate (pH 5.2–5.6) containing 0.04% methylene blue for 5–10 min at room temperature and destained in 30% ethanol to visualize the

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PII: S0014-5793(99)01008-X

RNA. Mouse multi-tissue Northern blots were obtained from Clontech. Membranes were prehybridized, hybridized, washed, and exposed on X-ray films according to standard procedures. The bands were quantitated by densitometry using a Pharmacia-LKB instrument. For reprobing membranes were stripped by boiling in 0.5% SDS for 10 min.

2.3. Islet RNA RT-PCR analysis

Islets were isolated as described [13] and homogenized in TRIzol reagent (Gibco BRL) in the presence of 5 μg carrier *Escherichia coli* tRNA (Boehringer Mannheim). The total RNA was treated by RNase-free DNase (Boehringer Mannheim). Reverse transcription was done using Expand reverse transcriptase (Boehringer Mannheim). 2.5% of the cDNA from one pancreas-equivalent was amplified by PCR during 36 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 2.5 min.

2.4. Screening of cDNA arrays

Mouse gene discovery arrays (GDA) were obtained from Genome Systems Inc. (St. Louis, MO, USA). The arrays contained 18 378 individual genes double-spotted on 22×22 cm nylon filters, as well as 30 controls of *Arabidopsis* and *Drosophila* genes. The membranes were hybridized with probes prepared from RNA obtained from growing and growth-arrested βTC-tet cells. 2.5 μg poly(A)$^+$ RNA prepared as above was mixed with the control RNAs and reverse transcribed in the presence of [^{33}P]dCTP (NEN, specific activity 2000 Ci/mmol). Following hybridization and washes, the membranes were exposed onto a phosphorimager screen (Molecular Dynamics). The image was analyzed by Genome Systems software and normalized using the internal controls. Bacterial stabs containing individual clones from the IMAGE collection, which were used for detailed analyses, were obtained from Genome Systems.

2.5. Differential secretion and microsequencing

Proliferating and growth-arrested βTC-tet cells were washed twice in PBS and incubated in DMEM without serum for 24 h. The medium was collected, supplemented with a cocktail of protease inhibitors (Boehringer Mannheim), centrifuged for 5 min at 2000 rpm, and concentrated 10-fold on a Centriprep 3 column (cut-off 3000 Da, Amicon). The protein in the concentrate was precipitated by 80% cold acetone, followed by centrifugation for 5 min at 3000 rpm. Protein pellets were resuspended in Laemmli loading buffer, and samples of 35 μg protein were fractionated on a preparative 11% SDS-polyacrylamide gel. Protein bands were detected by staining with Coomassie blue. Differentially secreted protein bands were excised from the gel and subjected to protein microsequencing at the Protein Analysis Center, Technion, Haifa, Israel.

3. Results

3.1. Effects of growth arrest on mRNA levels of known β cell genes

Genes known to be expressed in β cells were analyzed for changes in expression as a result of growth arrest. We have previously reported a 3–5-fold increase in insulin content following a period of over 2 weeks growth arrest in the presence of tet [11]. As shown in Fig. 1, this increase is reflected in a progressive increase in insulin mRNA level, indicating that the higher insulin content results from increased insulin gene transcription or mRNA stability. In contrast, no significant changes were observed in the mRNA levels encoding other known proteins with major roles in insulin biosynthesis and secretion, such as the glucose phosphorylating enzyme glucokinase, the glucose transporter GLUT2, the insulin gene transcription factor Pdx1, and the proinsulin convertases PC1 and PC2 (data not shown).

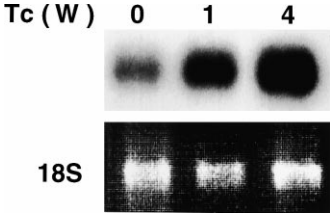

Fig. 1. Effect of growth arrest on insulin mRNA levels in βTC-tet cells. Cells at passage 14 were grown in the absence or presence of tet for the indicated number of weeks. One μg of total RNA was analyzed by Northern blot using an insulin cDNA probe. Ribosomal RNA served for loading normalization.

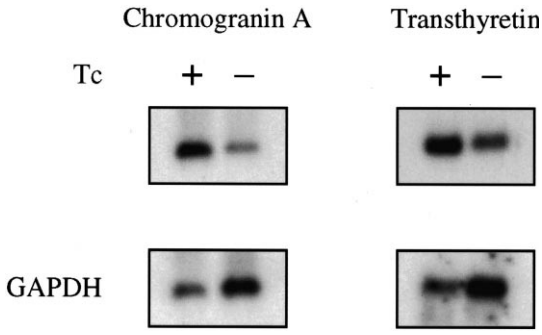

Fig. 2. Effects of growth arrest on expression of chromogranin A and transthyretin in βTC-tet cells. βTC-tet cells at passages 26–27 were grown in the absence or presence of tetracycline (Tc) for 10 days. Two μg of poly(A)$^+$ mRNA was analyzed by Northern blots with probes generated by RT-PCR amplification of βTC-tet cell mRNA using primers specific for mouse chromogranin A and transthyretin. The membranes were reprobed with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe to normalize for loading.

3.2. Identification of genes induced in βTC-tet cells by growth arrest using analysis of differentially secreted proteins

The insulin secretory vesicles contain, in addition to insulin, minor quantities of a number of proteins which participate in proinsulin maturation within the vesicle. In addition, β cells may secrete other proteins through the constitutive pathway. Culture media conditioned by proliferating or growth-arrested βTC-tet cells were compared for identification of changes in the profile of proteins secreted from the cells. Growing and growth-arrested cells were incubated in medium without serum for 24 h, and the conditioned medium was fractionated using preparative polyacrylamide-SDS gels, followed by Coomassie blue staining. This analysis revealed a total of about 36 different bands, eight of which were differentially secreted by proliferating and growth-arrested cells (data not shown). A number of the prominent differential bands were excised from the gel and subjected to microsequencing. The sequence obtained was compared to protein databases. One of the bands secreted at higher levels by growth-arrested cells was identified as insulin II, based on the amino acid sequence GFFYTPMSRR. Two other differentially secreted proteins were identified by this approach: chromogranin A, identified by the amino acid sequence PVNSPMTK, and transthyretin, identified by the amino acid sequences KVLDAVRGSPAVDVAVK and KSYWK. Chromogranin A is a constituent of the matrix of secretory vesicles in a variety of neuroendocrine cell types and plays a role in their maturation. Its expression has been demonstrated in normal islets from a variety of species [14,15]. Transthyretin is a retinol binding protein and a carrier of thyroid hormones in the circulation. It is expressed in the

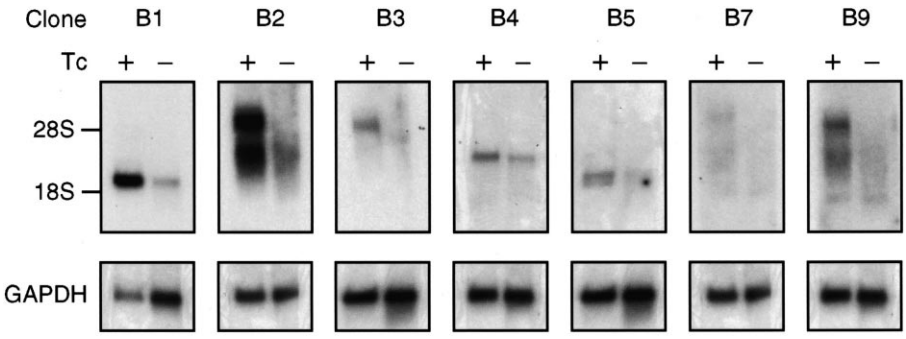

Fig. 3. Effect of growth arrest on expression of mRNAs for clones B1–B9 in βTC-tet cells. βTC-tet cells at passages 26–27 were grown in the absence or presence of tetracycline (Tc) for 10 days. One μg of poly(A)$^+$ mRNA was analyzed by Northern blots with probes generated from the plasmid inserts of the indicated clones. The membranes were reprobed with GAPDH to normalize for loading.

liver, choroid plexus, intestine and kidney. Its expression has been documented in normal islet cells, however its physiological role there remains unknown [16–18]. Northern blot analysis revealed a 3–4-fold increase in the levels of mRNAs encoding these two proteins in growth-arrested, compared with proliferating cells (Fig. 2), indicating that the change in secretion was associated with elevated transcription or increased mRNA stability.

Table 1
Genes induced in βTC-tet cells by growth arrest as detected by cDNA arrays

No.	Fold induction	EST number	Homology		
			%	Gene	Accession number
1 (B2)	21.93	W97235	97	Ganglioside-induced differentiation associated protein 10, mouse	Y17860
2 (B4)	20.14	W65819		–	
3 (B1)	12.17	AA008593	100	Synaptogyrin 3, mouse	AJ002309 (human)
4 (B5)	11.48	AA051515		–	
5 (B3)	8.23	AA120153		–	
6 (B8)	5.80	AA416122		–	
7 (B7)	5.57	AA416215	87	Serine-threonine kinase CPG16, rat	U78857
8 (B6)	5.35	AA064016		–	
9 (B9)	5.13	AA388088		–	
10	4.80	AA530020		–	
11	4.78	AA387252		–	
12	4.77	AA387120		–	
13	4.77	AA238652		–	
14	4.48	AA207992	100	Cysteinyl-tRNA synthetase, mouse	AB015589
15	4.37	AA388111		–	
16	4.36	AA177667	97	TIS7 protein, mouse	X17400
			96	IFRD1 (PC4), rat	J04511
17	4.24	AA444721		–	
18	4.20	AA051357		–	
19	4.00	AA286313	98	RNA binding protein, mouse	L17076
			98	RNA binding protein, mouse	S72641
20	3.99	AA117941		–	
21	3.95	AA048762		–	
22	3.85	AA014942		–	
23	3.79	W82580		–	
24	3.77	AA212269		–	
25	3.73	AA420104	91	KIAA0710 protein, human	AB014610
26	3.70	W63930		–	
27	3.67	AA474754	96	CDE-1 binding protein, mouse	U37485
			95	Amyloid precursor-like protein 2 isoform 751, mouse	U15571
28	3.33	W54048		–	
29	3.30	AA237377	88	Reticulon gene family protein RTN3, human	AF059524
30	3.28	AA123188	85	Translational activator GCN1, human	U88836
31	3.21	AA388147		–	
32	3.18	AA388068	92	KIAA0462 protein, human	AB007931
33	3.16	AA387118		–	
34	3.15	AA016373	100	TFIIH transcription/DNA repair factor p52, mouse	AF054823
35	3.14	AA437793	85	RNase H I, human	Z97029
36	3.06	AA174859		–	
37	3.02	W78447		–	
38	3.01	AA274605		–	

Genes shown are those induced >3-fold, with a minimum normalized intensity >1000 in growth-arrested cells, in descending order of fold induction. Clone numbers in parentheses were assigned to those clones analyzed in detail.

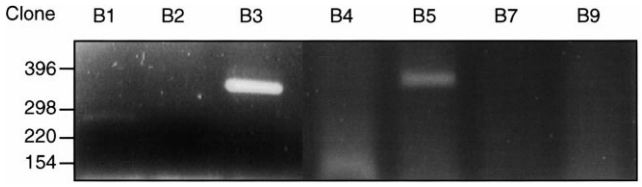

Fig. 4. Expression of mRNAs for clones B1–B9 in normal mouse islets. RNA extracted from isolated adult mouse islets was subjected to RT-PCR analysis with primers specific for each clone. Size markers are in bp.

3.3. *Identification of genes induced by growth arrest in βTC-tet cells using cDNA arrays*

To systematically survey the changes in gene expression between proliferating and growth-arrested cells, we compared the patterns of gene expression by hybridization of labeled cDNA, prepared from mRNA obtained from proliferating and growth-arrested cells, to mouse cDNA arrays. We used an array of over 18 000 non-redundant mouse cDNA clones from the IMAGE collection, consisting mostly of expressed sequence tags (ESTs). Tables 1 and 2 list the major changes in gene expression, in decreasing order of change magnitude. Thirty-eight genes were induced over 3-fold by growth arrest, 13 of which had high homology to known genes in GenBank (Table 1). Clone #3 (also denoted B1), synaptogyrin 3, is a component of the secretory vesicle membrane, with a possible role in vesicle trafficking [19]. The majority of the remaining known gene products do not have recognized functions in β cells. The other 25 genes with no homology in GenBank likely represent novel genes. The 3-fold induction cut-off line chosen here is arbitrary, and was used to focus attention on the genes at the top of the list of induced genes. It is conceivable that further data analysis of genes induced <3-fold will yield additional information on genes relevant to β cell biology. Search of the list of the top 1000 induced genes for known β cell genes revealed that GLUT2 was induced 1.74-fold. Glucokinase, hexokinase, GLUT1, PC1, and PC2 did not appear in the list of the top 1000 induced genes. Insulin and Pdx1 were not included in the cDNA array.

The list of genes downregulated by growth arrest at least 3-fold (Table 2) includes 16 genes, seven of which have high homology to known genes. This list is likely to include genes related to cell replication, as well as genes turned on in dividing β cells as part of the transformed phenotype. Clone #8 encodes a homolog of the proto-oncogene ret tyrosine kinase receptor [20], which may be involved in the regulation of β cell proliferation. The other nine genes without homology in GenBank are likely to represent novel genes.

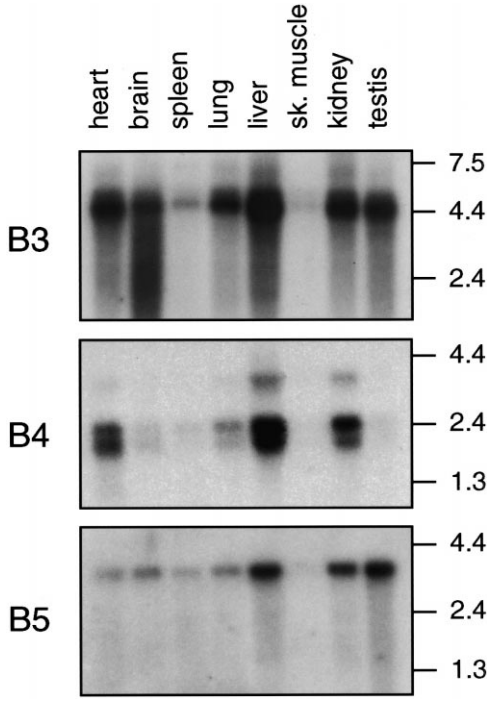

Fig. 5. Distribution of mRNAs for clones B3–B5 in various mouse tissues. Membranes containing 2 μg of poly(A)$^+$ mRNA were probed with inserts from clones B3–B5. Size markers are in kb.

The genes induced by growth arrest, which are likely to include genes related to β cell differentiation and function, were analyzed in more detail. We chose to focus initially on seven of the genes at the top of the list, labeled B1–B5, B7 and B9 (Table 1). Their expression in βTC-tet cells was analyzed

Table 2
Genes downregulated in βTC-tet cells by growth arrest as detected by cDNA arrays

No.	Fold reduction	EST number	Homology		
			%	Gene	Accession number
1	18.49	W30126		–	
2	17.80	AA049791		–	
3	17.08	AA450460		–	
4	11.29	AA498608	99	Peroxisomal integral membrane protein PMP34, mouse	AJ006341
5	10.37	AA124868	92	Plasminogen activator inhibitor 2, mouse	X16490
6	10.06	AA289542	98	Cathepsin B, exon 10, mouse	M65270
7	9.80	AA254672		–	
8	9.75	AA497635	100	ret-11, mouse	AB016784
9	9.44	AA416278	92	Zinc-finger DNA-binding protein, human	D45132
10	8.61	AA003640		–	
11	8.41	AA111647	83	MUF1 protein, human	X86018
12	8.34	AA475767		–	
13	7.87	AA048029		–	
14	7.11	AA119654	100	Hsp70-related NST-1, mouse	U08215
15	6.11	W11186		–	
16	3.33	AA404050		–	

Genes shown are those downregulated >3-fold, with a minimum normalized intensity >1000 in proliferating cells, in descending order of fold reduction.

by Northern blots, using probes containing partial cDNA sequence, which were obtained from the IMAGE Consortium collection. All of them were confirmed by this analysis to be induced at the mRNA level by growth arrest (Fig. 3). The extent of induction was essentially in accordance with that predicted by the cDNA array hybridization (Table 1). Four of these seven genes (B1, B3–B5) were found to be expressed in normal mouse islets by RT-PCR analysis (Fig. 4). The other three (B2, B7, B9) may represent genes turned on in β cells in the initial period following withdrawal from the cell cycle, which are not expressed in terminally differentiated adult β cells. The three genes expressed in normal islets which do not have homology in GenBank (B3–B5) were hybridized to Northern blots containing mRNA from various mouse tissues (Fig. 5). All three of them were found to be expressed in multiple tissues, demonstrating that they are not β cell-specific genes. Nevertheless, they may be involved in important β cell functions. B3 showed in all the tissues the same 5-kb transcript seen in the βTC-tet cells. B4 had three transcripts in all tissues, indicating alternative splicing or related transcripts from a family of genes cross-hybridizing with B4. Only the long 3.5-kb transcript was observed in βTC-tet cells. B5 showed a single transcript of about 3.5 kb in all tissues, compared with a doublet of about 2.7 and 2.3 kb in βTC-tet cells.

4. Discussion

Our findings demonstrate that growth arrest of βTC-tet cells stimulates the expression of a number of genes associated with β cell function, and therefore can be regarded as a model of β cell differentiation. Growth arrest resulted in a significant increase in the levels of mRNA encoding preproinsulin, as well as mRNA for a known component of the insulin secretory vesicles, chromogranin A. In addition, expression of synaptogyrin 3, shown to form part of the secretory vesicle membrane in neurons, was also induced by growth arrest in βTC-tet cells. These findings are consistent with the increased insulin content of growth-arrested cells, compared with proliferating cells [11]. It should be noted that this analysis reflects steady-state levels of mRNAs at certain time points (1–4 weeks) following growth arrest, rather than de novo synthesis. We have previously reported that the rates of de novo proinsulin biosynthesis are similar in proliferating and growth-arrested cells [11]. It is possible that the large increase in insulin protein biosynthesis occurs within the first days following growth arrest. The amounts of newly synthesized insulin needed subsequently to maintain the established differences in the steady-state levels of insulin stored in the cells may not differ much between proliferating and growth-arrested cells, given that only a small fraction of the stored insulin is released into the culture medium [11]. The insulin mRNA analysis was based on normalization of the total RNA loaded using ribosomal RNA, assuming that no significant changes in cellular content of ribosomal RNA occur between proliferating and growth-arrested cells. When poly(A)$^+$ mRNA was analyzed for other genes, hybridization with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was used for normalization. When compared with residual ribosomal RNA in the mRNA preparations, we have noticed a decrease in GAPDH mRNA levels in growth-arrested cells, compared with proliferating cells (Figs. 2 and 3). Thus, although GAPDH mRNA levels are shown, we relied primarily on loading of equal mRNA amounts derived from equal cell numbers.

To survey the complete spectrum of changes in gene expression induced upon growth arrest in this model, we used differential hybridization to mouse cDNA arrays. The results reveal a complex pattern of gene activation and shut-off, which supports the view that proliferating and quiescent β cells express a significantly different array of genes. Some of the genes expressed preferentially in the replicating cells may represent genes turned on by Tag, which are not expressed in normal β cell proliferation. In addition to the changes detected at the mRNA level, it is likely that numerous changes occur at the translational and post-translational levels, which cannot be detected by this approach. Although proliferating βTC-tet cells express insulin and secrete it in a correctly regulated fashion, these findings indicate that growth arrest can result in a phenotype that resembles more closely that of mature, terminally differentiated β cells. These findings have implications for the usage of transformed β cell lines in cell therapy of type 1 diabetes.

Most of the genes listed in Tables 1 and 2 have not been previously documented in β cells. As shown by the tissue blots in Fig. 5, many of them, including those turned on by growth arrest, may not necessarily represent β cell-specific genes but rather are likely to be expressed in a variety of other tissues, as is the case with the vast majority of genes expressed in β cells and involved in important β cell functions. Further characterization of the genes activated by growth arrest, in particular those without homology to known genes, may provide new insights into these functions. It would also be of interest to determine whether similar changes in gene expression are observed upon withdrawal of β cells from the cell cycle during normal islet development.

Acknowledgements: This work was funded by a grant from the Israel Science Foundation to S.E. This work was performed in partial fulfillment of the requirements for a Ph.D. degree of Dalit Milo-Landesman.

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