Original Research

Cellular mechanisms underlying failed beta cell regeneration in offspring of protein-restricted pregnant mice

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Abstract

Low birth weight and poor foetal growth following low protein (LP) exposure are associated with altered islet development and glucose intolerance in adulthood. Additionally, LP-fed offspring fail to regenerate their β-cells following depletion with streptozotocin (STZ) in contrast to control-fed offspring that restore β-cell mass. Our objective was to identify signalling pathways and cellular functions that may be critically altered in LP offspring rendering them susceptible to developing long-term glucose intolerance and decreased β-cell plasticity. Pregnant Balb/c mice were fed a control (C; 20% protein) or an isocaloric LP (8% protein) diet throughout gestation and C diet thereafter. Female offspring were injected intraperitoneally with 35 mg/kg STZ or vehicle on days 1 to 5 for each dietary treatment. At 30 days of age, total RNA was extracted from pancreatic tissue for microarray analysis using the Affymetrix GeneChip Mouse Genome 430 2.0. Gene and protein expression were quantified from isolated islets. Finally, β-cell proliferation was determined *in vitro* following REG1α treatment. The microarray data and GO enrichment analysis indicated that foetal protein restriction alters the early expression of genes necessary for many cell functions, such as oxidative phosphorylation and free radical scavenging. Expression of *Reg1* was upregulated following STZ, whereas protein content was decreased in LP + STZ islets. Furthermore, REG1α failed to stimulate β-cell proliferation *in vitro* in LP + STZ islets. Therefore, early nutritional insults may programme the Reg1 pathway resulting in a limited ability to increase β-cell mass during metabolic stress. In conclusion, this study implicates the Reg1 pathway in β-cell regeneration and describes altered programming of gene expression in LP offspring, which underlies later development of cell dysfunction and glucose intolerance in adulthood.

Keywords: Reg1, β-cell regeneration, maternal protein restriction, streptozotocin, microarray

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Introduction

β-cell mass is reduced and/or β-cell function is impaired in both type 1 and type 2 diabetes. Stimulation of endogenous regenerative mechanisms to restore β-cell mass is one strategy being actively pursued for treatment in diabetes. Rodent models of β-cell injury have demonstrated the capacity for β-cell regeneration. Administration of the β-cell specific toxin, streptozotocin (STZ), in single or multiple doses to neonatal rodents has been followed by rapid and significant regeneration.[1–3] However, in adult animals the response is blunted leading to diabetes later in life.[4,5] There is evidence to support both β-cell replication and precursor cell differentiation as contributing mechanisms of β-cell regeneration.[6–9]

Environmental factors, such as diet, are known to impact β-cell development and plasticity. In humans, poor foetal growth and low birth weight are associated with increased risk of impaired glucose tolerance,[10] type 2 diabetes[11] and the metabolic syndrome[12] in adult life. Previous work has examined the impact of maternal protein restriction (LP) during foetal life in mice on islet development and long term glucose homeostasis.[3] Offspring of LP-fed mice were characterized by a decreased birth weight, and specifically in females, an altered islet mass postnatally and an impaired glucose tolerance in adulthood. Additionally, neonatal offspring of LP-fed dams subsequently treated with multiple low doses of STZ were unable to regenerate their β-cell mass to control levels by day 30. In contrast, offspring of control

Table 1 Real-time PCR primers utilized

Target	Primer (5′-3′)	Reference no.
Insulin I	Forward: AGCCCCGGGGACCTTCAGAC	NM_008386
	Reverse: GCGGGTCGAGGTGGGCCTTA	
Insulin II	Forward: ACCATCAGCAAGCAGGAAGCCT	NM_008387
	Reverse: GAAGAGCAGGGCCAGCAGGG	
Glucagon	Forward: AGCTTGGGCCCAGGACACACT	NM_008100
	Reverse: CCAGCTGCCTTGCACCAGCA	
Cyclin D1	Forward: GCACCAGCCACACAGCGGTA	NM_007631
	Reverse: GATGTGGGCAGGCTGCAGGG	
Reg1	Forward: TCCTGGGCAACTGGGTCTCCT	NM_009042
	Reverse: GGGCATCACAGTTGTCA TCCTTCCA	
Reg3δ	Forward: TCCACGCATCAGCTGTCCCCA	NM_013893
	Reverse: AGCCCAGGTCTGTGGTTCCAG	
Cyclophilin A	Forward: ATGGTCAACCCCACCGTGT	NM_008907
	Reverse: TTCTGCTGTCTTTGGAACTTTGTC	
18 s	Forward: ACGATGCCGACTGGCGATGC	NR_003278
	Reverse: CCCACTCCTGGTGGTGCCCT	

(C) fed dams that received STZ were able to regenerate β-cell mass. There was a significant increase in duct-associated islet neogenesis in female C + STZ mice at d7 (Cox A.R., unpublished observations), while the female LP + STZ mice had an increased presence of PDX-1^+/insulin$^-$ putative precursor cells.[3] A lack of differentiation of this PDX-1^+/insulin$^-$ population into mature β-cells could contribute to the failure of regeneration. Programming by the LP diet may therefore reduce expression of initiating factors for β-cell regeneration.

The molecular signalling mechanisms involved in β-cell regeneration remain unresolved. Regeneration likely involves growth factor signalling to upregulate cell cycle genes and proliferation and/or differentiation signals initiating a cascade of transcription factor expression. The origin of these signals may include, but are not limited to, the islet vasculature,[13] the extracellular matrix,[13] α-cells,[1,14] and bone marrow-derived progenitor cells.[15] LP programming in rats has been shown to decrease expression of two important growth factors, VEGF and IGF-II.[16–18] Whether these or other factors influence β-cell regeneration in LP offspring is unknown.

Therefore, using DNA microarray analysis our first objective was to examine the expression profiles of C and STZ-treated offspring to identify genes critical to signalling in β-cell regeneration. Our second objective was to compare C and LP fed offspring following STZ to identify such candidate genes that may be altered under LP conditions, leading to a failure in β-cell regeneration. Furthermore, understanding how the intrauterine environment affects β-cell development could lead to intervention strategies to prevent glucose dysregulation and type 2 diabetes. Thus, our last objective was to identify signalling pathways and cellular functions that may be altered by LP exposure *in utero* based on altered gene expression profiles. Identifying novel factors capable of stimulating β-cell expansion could provide new therapies for β-cell replacement.

Materials and methods

Animals

Balb/c mice (Charles River Ltd, Montreal, QC, Canada) were housed in a temperature-controlled room with 12 h light:dark cycle. Water and food (Bio-Serv, Frenchtown, NJ) were given *ad libitum*. An isocaloric LP diet (8% protein) or control (C; 20% protein) diet was administered to pregnant mice during gestation as described previously.[3] The composition of the diets is shown in Supplemental Table 1, indicating no change in fat content between the C and LP diets, with an increase in the carbohydrate component to correct for calories. One day after birth until day (d) 5, female offspring received daily injections of either 35 mg/kg body weight of STZ (Sigma Chemical, St. Louis, MO) in sodium citrate buffer (0.1 mol/L, pH 4.5) or equal volume of the vehicle (sham). Female offspring were divided into four groups: C = control diet, sham injected; C + STZ = control diet, STZ injected; LP = low protein diet, sham injected; LP + STZ = low protein diet, STZ injected. On d 7, 14, and 30 female offspring were euthanized and the pancreas was removed and stored in RNAlater (Applied Biosystems Canada, Streetsville, ON, Canada) at −80°C. All animal procedures were approved by the Animal Care Committee of Western University in accordance with the guidelines of the Canadian Council for Animal Care.

Islet isolation

Female offspring were euthanized on d7 and islets were isolated by a modified protocol as previously described.[19] Briefly, pancreata from two to four female mice from each litter were removed and perfused in a Petri dish with 1 mL of Collagenase type V (1 mg/mL; Sigma) in Hank's Buffered Salt Solution (HBSS; Invitrogen Canada Inc., Burlington, ON, Canada). Pancreata were collected in 4–8 mL of HBSS + Collagenase type V and placed in a 37°C shaking water bath for 20–30 min. The digested tissue was passed through a 14 gauge needle and washed in HBSS + 5% bovine serum (Invitrogen), then centrifuged. Islets were separated from acinar and duct cells using a Dextran (MP Biomedicals, Solon, OH) gradient consisting of 27, 23, and 11% concentrations, then centrifuged for 25 min at 1700 rpm. Islets were collected from the 23/11% boundary, washed, and picked in HBSS + 5% serum. Islets for RNA use were stored in RNALater (Applied Biosystems) at −80°C and islets for protein use were sonicated in lysis buffer and stored at −80°C.

Microarray analysis

Total RNA was extracted and purified from whole pancreas of d30 female offspring using the RNeasy Plus Mini kit (Qiagen). Separate RNA samples were extracted from three pancreata per group, with each sample hybridized to a separate GeneChip ($n = 3$). All GeneChips were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://

www.lrgc.ca). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA).

For expression analysis, the Affymetrix (Santa Clara, CA) GeneChip Mouse Genome 430 2.0 (MOE430 2.0) array was used, containing 45,000 probe sets representing transcripts and variants from 34,000 mouse genes. All procedures, including cRNA synthesis, labelling, and hybridization to Affymetrix MOE430 2.0 GeneChips, were performed as described in the Affymetrix Technical Analysis Manual. GeneChips were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix).

Probe level data from the .CEL files were analysed using Partek Genomics Suite v6.5 (Partek, St. Louis, MO). Probes were imported and summarized using robust multi-array averaging and ANOVA was used to determine fold changes and *P* values. Differentially expressed genes between the four groups were selected based on fold change ($\geq$1.5) differences and a P value ≤ 0.05. Gene Ontology (GO) enrichment was performed within Partek to determine enriched GO terms (molecular functions, biological processes or cellular components) within a specific list of genes. A Fisher's exact test was used to determine significantly enriched GO terms. Affymetrix microarray expression data have been deposited in the Gene Expression Omnibus database (accession # GSE38215).

Reverse transcription and real-time PCR

Total RNA was extracted from whole pancreas from 7 -, 14 -, and 30-day-old female offspring and from isolated islets of d7 mice with the RNeasy Plus Mini kit (Qiagen). RNA quality was assessed as described above. RNA was treated with DNase (Invitrogen) to remove contaminating DNA. RNA from whole pancreas (4 μg) or isolated islets (73 ng) was reverse transcribed (RT) using Superscript III Reverse Transcriptase (Invitrogen) and random hexamers (Invitrogen). Primer sets directed against insulin I, insulin II, glucagon, *Ccnd1, Reg1*, *Reg3δ*, and the housekeeping gene *18 s* were designed using NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/; National Center for Biotechnology Information, Bethesda, MD) (Table 1). Primers were designed to have a melting temperature ~60°C, a product size between 80 and 150 bases, 4–5 base mismatches with similar non-target sequences with at least 1 near the 3′ end, and spanning an exon-exon junction when possible. Cyclophilin A primer sequences were designed as previously described.[20]

The relative abundance of each RNA transcript was determined by quantitative real-time RT-PCR (qPCR) using the Bio-Rad CFX384 real-time PCR machine (Bio-Rad, Mississauga, ON, Canada) with SYBR Green (Bio-Rad) for detection of PCR products. All primer sets were demonstrated to have good linear correlation and equal priming efficiency for an input cDNA dilution series compared with their cycle threshold (Ct) values. The relative abundance of each target within the treatment groups compared with the control was determined by the comparative Ct method using $2^{-\Delta\Delta Ct}$.

Western blot analysis

At least 200 islets isolated from 7-day-old female mice were sonicated in lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Igepal (MP Biomedicals), 1 mM phenylmethanesulfonylfuoride, 0.1% Aprotinin (Sigma), 1 × complete, mini protease inhibitor cocktail (Roche, Mississauga, ON, Canada) in deionized water and stored at −80°. Protein quantification was performed using a micro BCA protein assay kit (Pierce Protein Researcher, Rockford, IL) according to manufacturer's instructions. Protein samples were concentrated by acetone precipitation and then resuspended in 50 μL of sample buffer. Protein ($\leq$5 μg) was resolved on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen). Membranes were probed with a sheep anti-REG1 antibody (1 : 200; R&D Systems, Minneapolis, MN) with a donkey anti-sheep secondary (1 : 1000; R&D Systems). Protein detection was achieved with chemiluminescence (Pierce). Membranes were stripped and re-probed with a rabbit anti-β-actin antibody (1 : 5000, Abcam, Cambridge, MA), used as a loading control, with a goat anti-rabbit secondary antibody (1 : 10,000; Santa Cruz Biotechnology, Santa Cruz, CA). Image capture and densitometry was performed using GeneSnap v7.09 and GeneTools v 3.06 software (SynGene, Cambridge, England).

Reg1α islet treatment

Isolated islets were incubated overnight in 2 mL DMEM/F12 (Sigma) with 5% foetal calf serum and 5 μL/mL penicillin-streptomycin, and 0.5 μL/mL fungizone. Up to 110 islets were incubated for 72 h in media containing 0 or 10 nM REG1α (GenWay Biotech, San Diego, CA). One μL EdU (Invitrogen) was added for the final 6 h of incubation. Islets were picked and washed in HBSS, then treated with trypsin for 5 min at 37°C to generate a single cell suspension. Glass bottom dishes (Cedarlane, Burlington, ON, Canada) were treated with Cell Tak (BD Biosciences, Mississauga, ON, Canada) in sodium bicarbonate according to manufacturer's instructions. Cells were washed, then plated and fixed to dishes with 4% paraformaldehyde. EdU staining was performed with Click-it EdU cell proliferation assay (Invitrogen) according to manufacturer's instructions. Cells were blocked using Background Sniper (Biocare Medical, Concord, CA) for 5 min, followed by overnight incubation with mouse anti-insulin primary antibody (1 : 2000, Sigma) and 2 h incubation with Alexafluor 488 goat anti-mouse secondary antibody (1 : 500; Invitrogen). Twenty-five fields of view were analysed per dish, and the percentage of EdU-positive, insulin-positive β-cells were quantified.

Statistical analysis

Significance was determined by a Student's *t*-test or a two-way ANOVA with Bonferroni post-test using GraphPad Prism v5 software (GraphPad Software, La Jolla, CA). Statistical analysis of microarray data was performed as detailed above (Methods). All values are means ± SEM and significance level $P < 0.05$.

Results

Microarray analysis

DNA microarray technology was utilized to identify pancreatic genes involved in the metabolic deficiencies observed in female LP offspring, and in β-cell regeneration following STZ treatment. The impact of foetal LP exposure was greater in female offspring compared with males, evidenced by altered β-cell mass and glucose intolerance,[3] thus current studies focused on females only. Comparisons were made between groups (C + STZ versus C; LP versus C; LP + STZ versus LP; LP + STZ versus C + STZ) to create gene lists containing all genes with a significant ($P < 0.05$) change in expression of ≥ 1.5 fold (Supplemental Tables 2–5). Based on these criteria, 369 transcripts were significantly reduced ($P < 0.05$) in the LP offspring compared to control (C)-fed animals and 35 transcripts were significantly increased ($P < 0.05$; Supplemental Table 2). Animals in the C + STZ group, which were capable of regeneration of β-cells by day 30,[3] had a significantly decreased ($P < 0.05$) expression of 60 transcripts, while 18 transcripts were significantly increased ($P < 0.05$) by ≥ 1.5 fold compared to the sham-injected controls (Supplemental Table 3). Only nine transcripts were significantly decreased ($P < 0.05$) in the LP + STZ group compared to LP alone by ≥1.5 fold (Supplemental Table 4). This suggests that STZ treatment does not have a large additive affect on gene expression in LP offspring. However, comparing LP + STZ to C + STZ mice, 411 transcripts were significantly decreased ($P < 0.05$) and 15 transcripts were significantly increased ($P < 0.05$; Supplemental Table 5). These represent candidate genes that might be linked to the failure of β-cell plasticity observed in LP-fed animals following STZ treatment.

GO enrichment

Using the gene lists (Supplemental Tables 2–5) created from the microarray data, GO enrichment analysis was performed as an indication of which biological processes, molecular functions, or cellular components may be affected by prior LP exposure. The GO enrichment data was mined to identify cellular systems affected by LP exposure that may predispose for the development of altered glucose metabolism and β-cell function.

The gene list comparing the C and LP groups consisted of many GO terms relating to cellular respiration, such as the electron transport chain (ETC) and ATP synthesis coupled proton transport (Table 2). These GO terms were significantly enriched as indicated by the respective *P* value. This suggests that LP offspring have significant alterations to transcripts that may have an important impact on cellular respiration. The gene list comparing fold change between C + STZ and LP + STZ was also significantly enriched for GO terms relating to cellular respiration (Table 2). A greater percentage of genes from the C + STZ versus LP + STZ gene list were present within each GO term compared to the C versus LP list.

Table 2 Enrichment of GO terms relating to cellular respiration

		LP versus control gene list			LP + STZ vs C + STZ gene list		
Function	**Type**	***P* value**	**% Genes in group**	**# Genes in group**	***P* value**	**% Genes in group**	**# Genes in group**
Cytochrome-c oxidase activity	Molecular function	2.50 E-07	37.50	6	2.24 E-33	43.75	7
Proton-transporting ATP synthase complex, coupling factor F(o)	Cellular component	4.72 E-07	50.00	5	2.10 E-89	90.00	9
Electron transport chain	Biological process	1.46 E-06	11.46	11	3.76 E-61	25.53	24
Proton transport	Biological process	2.34 E-04	12.50	6	4.75 E-42	29.17	14
ATP synthesis coupled proton transport	Biological process	3.14 E-04	15.15	5	3.76 E-54	39.39	13
NADH dehydrogenase (ubiquinone) activity	Molecular function	3.44 E-03	17.65	3	3.62 E-16	29.41	5
NADH dehydrogenase (quinone) activity	Molecular function	3.44 E-03	17.65	3	3.62 E-16	29.41	5
ATP biosynthetic process	Biological process	2.00 E-01	4.44	2	4.98 E-11	15.56	7
Mitochondrial part	Cellular component	1.00 E + 00	7.08	24	3.71 E-47	12.76	43
Oxidation reduction	Biological process	1.00 E + 00	2.85	17	1.26 E-10	5.56	33

Using the microarray data, gene lists were created for significantly altered genes in the LP group compared to the C group with ≥1.5 fold change and also for the LP + STZ versus the C + STZ groups. These gene lists were analysed for GO terms that were significantly enriched based on a Fisher's exact test with $P < 0.05$. The percentage and number of genes from the gene list out of the total genes for that GO term are listed.

Table 3 Mitochondrial electron transport chain components

Probeset ID	Gene symbol	Gene name	C + STZ	LP	LP + STZ
Complex I					
1422241_a_at	Ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	−1.158	−1.568*	−1.94†
1417368_s_at	Ndufa2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2	−1.069	−1.459*	−1.62†
1452790_x_at	Ndufa3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3	1.004	−1.340*	−1.64†
1417286_at	Ndufa5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5	−1.024	−1.266*	−1.41†
1448427_at	Ndufa6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (B14)	1.544*	1.096	−1.17†
1428360_x_at	Ndufa7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7 (B14.5a)	1.125	−1.123	−1.39†
1423692_at	Ndufa8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8	−1.137	−1.266*	−1.36
1418068_at	Ndufa10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	1.131*	1.152*	1.15
1429708_at	Ndufa11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11	1.103	−1.220	−1.47†
1430713_s_at	Ndufa13	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	−1.116	−1.509*	−1.83†
1448483_a_at	Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	1.009	−1.383*	−1.80†
1416547_at	Ndufb3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3	−1.091	−1.276*	−1.51†#
1434056_a_at	Ndufb6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6	−1.224	−1.753*	−1.95†
1416417_a_at	Ndufb7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7	−1.006	−1.314*	−1.49†
1448198_a_at	Ndufb8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8	−1.215	−1.540*	−1.78†
1452184_at	Ndufb9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9	−1.190	−1.639*	−1.86†
1416056_a_at	Ndufb11	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11	−1.098	−1.611*	−1.850†
1448284_a_at	Ndufc1	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1	1.554	1.110	−1.19†
1423737_at	Ndufs3	NADH dehydrogenase (ubiquinone) Fe−S protein 3	1.101	−1.138	−1.33†
1433603_at	Ndufs6	NADH dehydrogenase (ubiquinone) Fe-S protein 6	−1.060	−1.354*	−1.575†
1424313_a_at	Ndufs7	NADH dehydrogenase (ubiquinone) Fe-S protein 7	1.044	−1.258	−1.434†
1428179_at	Ndufv2	NADH dehydrogenase (ubiquinone) flavoprotein 2	1.095	−1.225	−1.52†
1424628_a_at	Ndufv3	NADH dehydrogenase (ubiquinone) flavoprotein 3	−1.156	−1.499*	−1.83†
Complex II					
1418005_at	Sdhb	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	−1.079	−1.292	−1.52†
Complex III					
1453229_s_at	Uqcrh	ubiquinol-cytochrome c reductase hinge protein	1.101	−1.322*	−1.582†
1448292_at	Uqcr	ubiquinol−cytochrome c reductase (6.4kD) subunit	1.259	−1.157	−1.43†
Complex IV					
1448322_a_at	Cox4i1	cytochrome c oxidase subunit IV isoform 1	−1.088	−1.538*	−1.758†
1415933_a_at	Cox5a	cytochrome c oxidase, subunit Va	−1.164	−1.636*	−1.73†
1416902_a_at	Cox5b	cytochrome c oxidase, subunit Vb	−1.042	−1.396*	−1.612†
1417417_a_at	Cox6a1	cytochrome c oxidase, subunit VIa, polypeptide 1	−1.214	−1.697*	−1.83
1416565_at	Cox6b1	cytochrome c oxidase, subunit VIb polypeptide 1	−1.082	−1.432*	−1.69†
1434491_a_at	Cox6c	cytochrome c oxidase, subunit VIc	−1.217	−1.586*	−2.06†#
1416970_a_at	Cox7a2	cytochrome c oxidase, subunit VIIa 2	−1.076	−1.573*	−1.98†
1429188_at	Cox11	COX11 homolog, cytochrome c oxidase assembly protein (yeast)	−1.137	−1.360*	−1.497
Complex V					
1416058_s_at	Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	−1.087	−1.336	−1.56†
1423716_s_at	Atp5d	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	1.374*	1.020	−1.137†
1416567_s_at	Atp5e	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	−1.037	−1.467*	−1.791†
1415980_at	Atp5g2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c2 (subunit 9)	1.160	−1.110	−1.34†
1454661_at	Atp5g3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c3 (subunit 9)	−1.004	−1.459*	−1.57†
1423676_at	Atp5h	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	−1.163	−1.536*	−1.77†
1416143_at	Atp5j	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f	−1.055	−1.503*	−1.87†
1416269_at	Atp5j2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2	1.122	−1.310	−1.675†
1450640_x_at	Atp5k	ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e	−1.112	−1.487*	−1.89†
1448203_at	Atp5l	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g	1.115	−1.257	−1.60†
1417970_at	Atp5s	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit s	−1.120	−1.249*	−1.244
1416278_a_at	Atp5o	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	1.107	−1.283	−1.52†

Represents fold change in expression versus the control group for microarray analysis performed using total RNA samples from d30 pancreata of female offspring from C- and LP-fed dams, with or without subsequent STZ treatment. Data were analysed using a two-way ANOVA ($n = 3$). *$P < 0.05$ versus C + sham; †$P < 0.05$ versus C + STZ; #$P < 0.05$ versus LP + sham.

Table 4 Genes encoding antioxidants and related proteins

Probeset ID	Gene symbol	Gene name	C + STZ	LP	LP + STZ
1460671_at	Gpx1	glutathione peroxidase 1	1.048	−1.365*	−1.594†
1449106_at	Gpx3	glutathione peroxidase 3	−1.191	−1.359*	−1.34
1451695_a_at	Gpx4	glutathione peroxidase 4	−1.160	−1.468*	−1.72†
1441931_x_at	Gss	glutathione synthetase	1.145	1.750*	1.864†
1451124_at	Sod1	superoxide dismutase 1, soluble	1.028	−1.258*	−1.446†
1417634_at	Sod3	superoxide dismutase 3, extracellular	1.249*	1.219*	1.174
1416000_a_at	Prdx1	peroxiredoxin 1	−1.110	−1.541*	−1.526
1418506_a_at	Prdx2	peroxiredoxin 2	−1.150	−1.322*	−1.43
1416167_at	Prdx4	peroxiredoxin 4	−1.281	−1.696*	−2.022†
1416381_a_at	Prdx5	peroxiredoxin 5	1.050	−1.102	−1.301†
1452823_at	Gstk1	glutathione S-transferase kappa 1	1.024	−1.052	−1.280†#
1422072_a_at	Gstm6	glutathione S-transferase, mu 6	1.014	−1.043	−1.133†#
1449575_a_at	Gstp1	glutathione S-transferase, pi 1	−1.189	−1.550*	−1.682
1418186_at	Gstt1	glutathione S-transferase, theta 1	−1.092	−1.123	−1.35†#
1415897_a_at	Mgst1	microsomal glutathione S−transferase 1	−1.348	−1.559*	−1.91

Represents fold change in expression versus the control group for microarray analysis performed using total RNA samples from d30 pancreata of female offspring from C- and LP-fed dams, with or without subsequent STZ treatment. Data were analysed using a two-way ANOVA ($n = 3$). *$P < 0.05$ versus C + sham; †$P < 0.05$ versus C + STZ; #$P < 0.05$ versus LP + sham.

Table 5 Islet hormones, processing and secretion

Probeset ID	Gene symbol	Gene name	C + STZ	LP	LP + STZ
1422447_at	Ins1	insulin I	−4.959*	−2.434*	−4.063#
1422446_x_at	Ins2	insulin II	−3.285*	−2.124*	−3.570#
1425952_a_at	Gcg	Glucagon	−1.764	−2.968*	−2.092
1417954_at	Sst	Somatostatin	−1.447	−1.812*	−1.54
1423510_at	Iapp	Islet amyloid polypeptide	−5.323*	−3.439*	−4.385
1449067_at	Slc2a2/Glut2	glucose transporter 2	−1.912*	−1.702*	1.894
1448312_at	Pcsk2/PC2	proprotein convertase 2	−1.678*	−1.648*	−1.52
1423529_at	G6pc2	glucose-6-phosphatase, catalytic, 2	−5.254*	−3.687*	−3.996
1415949_at	Cpe	carboxypeptidase E	−1.692*	−1.756*	−2.055
1450708_at	Scg2	secretogranin II	−2.024*	−1.795*	−1.795
1423150_at	Scg5	secretogranin V	−1.801	−1.704	−1.760
1418149_at	Chga	chromogranin A	−1.702*	−1.445	−1.698
1448698_at	Ccnd1	cyclin D1	−1.080	−1.099*	−1.12
1424942_a_at	c-Myc	myelocytomatosis oncogene	1.594*	1.175	−1.033†
1415905_at	Reg1	regenerating islet-derived 1	−1.159	−1.441*	−1.559
1448290_at	Reg3β	regenerating islet-derived 3 beta	1.121	−1.551	−1.36
1424009_at	Reg3δ	regenerating islet-derived 3 delta	−1.080	−1.299	−1.524
1419666_x_at	Nupr1	nuclear protein 1	−1.048	−1.286	−2.147†#
1435064_a_at	Tmem27	transmembrane protein 27	−1.997*	−2.495*	−2.078
1452014_a_at	Igf-1	insulin-like growth factor 1	−1.010	−1.253*	−1.511†
1443969_at	Irs2	insulin receptor substrate 2	1.111	1.181*	1.149
1422174_at	Pdx1	pancreatic and duodenal homeobox 1	−1.237*	−1.183*	−1.212
1421112_at	Nkx2.2	NK2 transcription factor related, locus 2	−1.072	1.031	−1.076#
1448266_at	Edf1	endothelial differentiation-related factor 1	1.135	−1.226	−1.248†
1443803_x_at	Hoxa5	homeobox A5	1.299	2.242	2.215†
1416072_at	Cd34	CD34	−1.413	−1.606*	−1.547

Represents fold change in expression versus the C group for microarray analysis performed using total RNA samples from d30 pancreata of female offspring from C- and LP-fed dams, with or without subsequent STZ treatment. Data were analysed using a two-way ANOVA ($n = 3$). *$P < 0.05$ versus C + sham; †$P < 0.05$ versus C + STZ; #$P < 0.05$ versus LP + sham.

Electron transport chain

GO enrichment suggested an impact of prior LP exposure on cellular respiration, thus we identified transcripts encoding ETC enzymes as an indication of potential mitochondrial dysfunction (Table 3). Notably, 23 transcripts related to Complex I of the ETC were significantly altered ($P < 0.05$), the majority of which were downregulated in the LP and/or LP + STZ groups. ATP synthase (Complex V) is the final step in the ETC leading to the production of ATP, and 12 transcripts encoding for ATP synthase subunits were also significantly downregulated ($P < 0.05$) in the LP and/or LP + STZ groups. Similar observations were also found for transcripts encoding subunits of Complex II, III and IV. Decreased expression of ETC enzyme encoding transcripts may result in mitochondrial dysfunction in LP offspring.

Antioxidant expression

The mitochondrial respiratory chain is a major site of reactive oxygen species (ROS) production which can be detrimental to cell function and survival.[21,22] Given the impact of LP on ETC gene expression, we identified genes responsible for ROS scavenging in the microarray (Table 4). Expression of transcripts encoding for the antioxidants glutathione peroxidase (*Gpx1*, *Gpx3*, *Gpx4*), peroxiredoxin (*Prdx1*, *Prdx2*, *Prdx4*) and glutathione S-transferase (*Gstp1*, *Mgst1*) were significantly decreased ($P < 0.05$) in the LP group compared to control. Similarly, in the LP + STZ group there was a significant decrease ($P < 0.05$) in expression of transcripts for antioxidants (*Gpx1*, *Gpx4*, *Prdx4*, *Prdx5*, *Gstk1*, *Gstm6*, *Gstt1*) compared to the C + STZ group, while expression of the glutathione S-transferase members (*Gstk1*, *Gstm6*, *Gstt1*) were further downregulated ($P < 0.05$) compared to the LP group. Interestingly, the expression of the enzyme involved in glutathione synthesis, glutathione synthetase (*Gss*), was significantly upregulated ($P < 0.05$) in the LP and LP + STZ groups compared to the C and C + STZ groups, respectively. Transcripts encoding for superoxide dismutase (SOD), which converts superoxide into hydrogen peroxide and water, were differentially expressed; *Sod1* was significantly downregulated ($P < 0.05$) in the LP and LP + STZ groups while *Sod3* was significantly upregulated ($P < 0.05$) in the C + STZ and LP groups.

Islet function

We identified genes encoding the islet hormones, enzymes for hormone processing, and glucose transporters in the microarray data to determine if early changes in gene expression underlie the later development of glucose intolerance (Table 5). Expression of the insulin genes and islet amyloid polypeptide were significantly decreased ($P < 0.05$) in both the C + STZ and LP groups compared to control, with a further decrease ($P < 0.05$) in insulin expression in the LP + STZ group. Glucagon and somatostatin were significantly decreased ($P < 0.05$) in the LP group compared to control. Genes involved in glucose transport (*Glut2*), gluconeogenesis (*G6pc2*), and hormone processing (*Pc2, Cpe, Scg2*) were significantly decreased ($P < 0.05$) in the C + STZ and LP groups. An additive effect of LP and STZ was not observed, except for insulin I and II genes, which corresponds with the reduced β-cell mass at this time.[3]

β-Cell regeneration

Microarray analysis was performed on whole pancreas to examine both local islet specific changes in gene expression, as well as extra-islet changes that may contribute to neogenesis or stimulation of β-cell growth. Genes encoding known regulators of the cycle, mitogenic and transcription factors were investigated in the microarray data (Table 5). Expression of the important cell cycle gene, *Ccnd1*, was significantly reduced ($P < 0.05$) in the LP group compared to control. The proto-oncogene, *c-Myc*, was found to be significantly increased ($P < 0.05$) in the C + STZ group versus control, and also when compared to the LP + STZ group. The regenerating islet-derived gene, *Reg1*, was significantly decreased ($P < 0.05$) with prior LP exposure, while *Reg3β* and *Reg3δ* were unchanged. *Igf-I* was significantly downregulated ($P < 0.05$) in the LP and LP + STZ groups compared to the control and C + STZ groups, respectively. However, the expression of insulin receptor substrate 2 (*Irs2*), the downstream signalling target in the IGF-I and insulin signalling pathways, was significantly increased ($P < 0.05$) in LP offspring. Important β-cell transcription factors were also significantly altered ($P < 0.05$); *Pdx-1* was decreased in C + STZ and LP groups, while *Nkx2.2* was decreased in the LP + STZ group compared to LP alone. Also of note was a significant decrease ($P < 0.05$) in the expression of the hematopoietic and endothelial progenitor cell marker, *Cd34*, in LP offspring.

RNA measurements by real-time PCR

Quantitative real-time RT-PCR (qPCR) was performed on d30 pancreatic RNA samples to compare with the initial findings from microarray analysis. Day 7 and 14 samples were also analysed to examine a time course of changes in expression during β-cell regeneration. Insulin I, insulin II, and glucagon were selected on the basis that altered islet mass may impact their gene expression. *Ccnd1* is a prime regulator of the cell cycle, with *Reg1* and *Reg3δ* as potential regulators of cell cycle genes, which were also selected as candidates. Gene expression for insulin I and II was significantly increased ($P < 0.05$) in C + STZ and LP offspring compared to control at d7, while in LP + STZ mice, insulin II was significantly greater ($P < 0.05$) than C + STZ mice (Figure 1a,b). By d14, insulin I gene expression was unaltered between groups; however, insulin II expression was significantly reduced ($P < 0.05$) with STZ and LP treatment. At d30 both insulin genes were significantly upregulated ($P < 0.05$) in the LP offspring compared to controls, while the LP + STZ group had a significant reduction ($P < 0.01$) compared to the untreated LP offspring. Glucagon gene expression was significantly increased ($P < 0.01$) at d7 in the LP offspring versus the control but no other changes were observed during the time of study (Figure 1c). The expression of *Ccnd1* at d7 was significantly reduced ($P < 0.05$) for the C + STZ and LP groups versus control (Figure 1d). Prior LP exposure had a significant effect

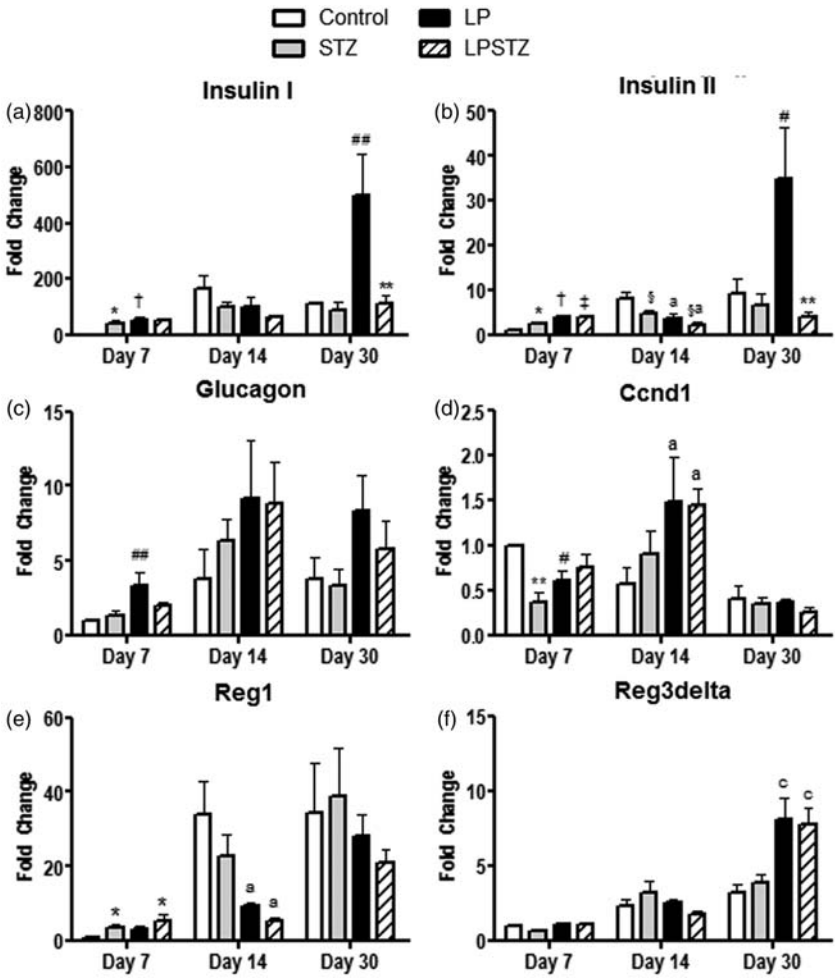

Figure 1 Gene expression changes in whole pancreas following LP diet or STZ treatment. Fold change in gene expression compared to d7 C determined by qPCR for the C (white bars), C + STZ (grey bars), LP (black bars) and LP + STZ (hatched bars) groups at d7, 14 and 30 were calculated using the comparative cycle threshold (Ct) method, $2^{-\Delta\Delta Ct}$, with cyclophilin A used as a reference gene. (a) insulin I, (b) insulin II, (c) glucagon, (d) *Ccnd1*, (e) *Reg1*, (f) *Reg3delta (δ)*. Fold change ± SEM; $n = 6$/group. Data were analysed using a 2-way ANOVA with Bonferroni's post-test within each time point. *$P < 0.05$ versus sham; ** $P < 0.01$ versus sham; # $P < 0.05$ versus C + sham; ## $P < 0.01$ versus C + sham, † $P < 0.001$ versus C + sham; ‡ $P < 0.05$ versus C + STZ; § $P < 0.05$ effect of STZ; a $P < 0.05$ effect of LP; c $P < 0.001$ effect of LP

($P < 0.05$), increasing expression of *Ccnd1* at d14 (Figure 1d). Initially *Reg1* was significantly upregulated ($P < 0.05$) with STZ treatment at d7, while LP exposure had an effect of significantly lowering ($P < 0.05$) expression at d14 (Figure 1e). For *Reg3δ* no changes were observed until d30 at which time prior LP exposure resulted in a significant increase ($P < 0.001$) in expression (Figure 1f). Interpretation of expression for β-cell specific genes should consider that β-cell mass was decreased with STZ treatment at days 7 and 14, and also at day 30 in the LP + STZ group.

While *Reg1* is normally produced by the pancreatic acinar cells,[23,24] it is known to be upregulated in regenerating islets.[25] Therefore, we analysed islet specific changes in RNA expression as a potential stimulus for β-cell regeneration at d7 from isolated islets. Similar to observations in the whole pancreas, *Reg1* gene expression was significantly increased ($P < 0.05$) with STZ treatment in islets, with a 5-fold increase in LP + STZ islets when compared to the LP sham group (Figure 2a). The other candidate genes, *Ccnd1* and *Reg3δ*, were expressed at low or undetectable levels within isolated islets (data not shown). These genes are widely expressed within many different cell types and therefore the changes observed from whole pancreatic RNA samples likely represent extra-islet changes in expression.

Western blot analysis

Protein lysates were collected from isolated islets at d7 and analysed to correlate the observed changes in REG1 gene expression with protein expression. Western blot analysis demonstrated a reduced presence of REG1 protein expression following STZ in LP offspring, which significantly differed from the C + STZ ($P < 0.01$) and LP sham ($P < 0.05$) groups (Figure 2b).

Reg1 and β-cell proliferation

To determine if reduced REG1 protein levels are related to the reduced β-cell regeneration in LP + STZ mice, we stimulated islets with REG1α *in vitro* (Figure 3). Proliferation

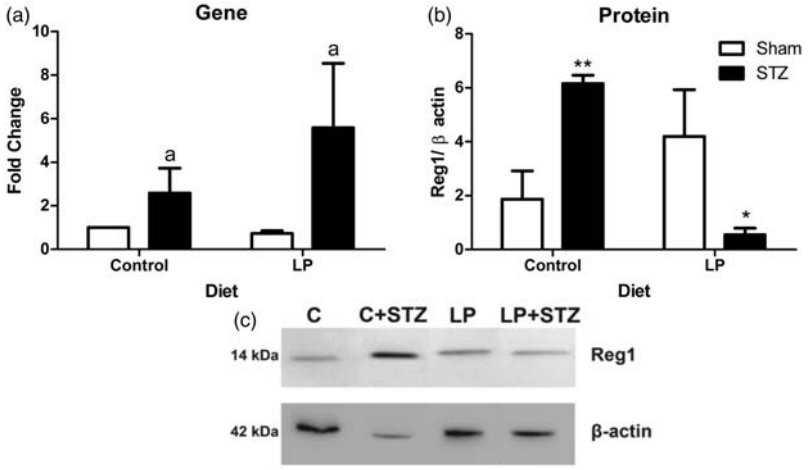

Figure 2 Reg1 gene and protein expression changes in isolated islets at d7. Islets were isolated at d7 from female offspring of C and LP fed mice with or without subsequent STZ administration. (a) Fold change in *Reg1* gene expression compared to the C group by qPCR, calculated using the ΔΔCt method with 18 s used as a reference gene. (b) REG1 protein expression levels were determined by western blot analysis from lysates of d7 isolated islets. Densitometry was performed for quantification of REG1 and normalized to β-actin levels. (c) Representative western blot image is shown. Top panel REG1 (14 kDa), and lower panel β-actin (42 kDa). White bars = sham, black bars = STZ. Means ± SEM; $n = 3$–5 for (a) and $n = 3$–6 for (b). Data were analysed using a two-way ANOVA with Bonferroni's post test. a, $P < 0.05$ effect of STZ. * $P < 0.05$ versus LP + sham; ** $P < 0.01$ versus LP + STZ

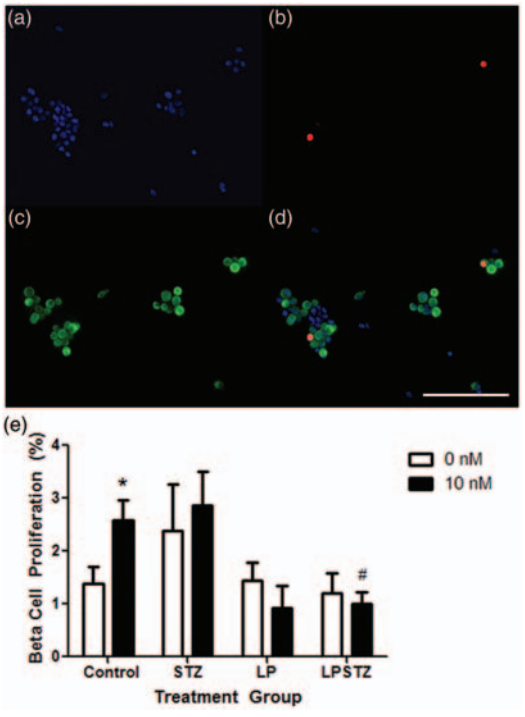

Figure 3 Reg1a stimulated β-cell proliferation. Isolated islets from d7 female offspring of C and LP fed mice with or without subsequent STZ administration were treated with 0 nM (white bars) or 10 nM (black bars) human Reg1a for 72 h. EdU was added to the cultures for the final 6 h of the experiment. Islets were dispersed and stained for (a) DAPI, (b) EdU, (c) insulin, with the merged image shown in (d). (e) The percentage of EdU^+ $insulin^+$ cells out of the total $insulin^+$ population was quantified. Means ± SEM; $n = 4$–7. Data were analysed using a Student's *t*-test

rates were determined by the percentage of $EdU^+/insulin^+$ cells compared to the total $insulin^+$ cells counted (Figure 3a). In isolated islets from 7-day-old C-fed mice, REG1α stimulated a significant ($P < 0.05$) 2-fold increase in proliferation compared to untreated islets (Figure 3b). The β-cell proliferation rate was elevated in untreated C + STZ islets, and no further increase was achieved following exposure to exogenous REG1α, suggesting that endogenous Reg1 gene expression was already elevated, as found in Figure 2. The basal proliferation rates in islets from LP or LP + STZ mice did not differ from C-fed mice, and in both conditions were unresponsive to exogenous REG1α (Figure 3b), suggesting that prior exposure to LP diet altered subsequent β-cell responsiveness to REG1α. This was not due to high endogenous levels of REG1 since the islet content was actually reduced in islets from LP + STZ mice (Figure 2). Moreover, comparing the effects of REG1α on C + STZ and LP + STZ islets, proliferation is significantly increased ~3-fold (C + STZ = 2.86 ± 0.65%; LP + STZ = 1.01 ± 0.22%; $P < 0.05$).

Discussion

Offspring exposed to protein restriction during foetal life demonstrate reduced birth weight, long-term impairments in glucose tolerance, and type 2 diabetes.[3,10,11,26] Previously, we have shown that female LP mouse offspring have reduced body weights at d1, altered islet cell mass at d7 and 14, and develop glucose intolerance by d130.[3] Here we used global expression arrays at d30 to identify genes that may be altered in the pancreas by LP exposure leading to impaired glucose metabolism in adulthood. Prior LP exposure significantly decreased all islet hormones, as well as the vital transcription factor *Pdx1*. *Pdx1* is important for pancreatic β-cell development, and plays a significant role in β-cell function.[27–29] Previous studies have similarly found reductions in *Pdx1* expression with nutrition deficiencies in foetal life.[30,31] Reduced expression of *Pdx1* in early life may be indicative of future β-cell failure. The *Glut2* glucose transporter, a *Pdx1* target gene,[32] was down-regulated in LP offspring. Genes involved in hormone

processing and secretion (*Pcsk2, Cpe, Scg2, Chga*) were downregulated following LP. However, β-cell function in LP offspring, as measured by an intraperitoneal glucose tolerance test, was similar to the control group at d42.[3] It is possible that altered expression of islet hormones and β-cell functional genes begins as early as d30, contributing to the later development of glucose intolerance by d130.

Support has been emerging for the theory that reprogramming of mitochondrial function is a key adaptative strategy enabling the foetus to survive in an energy-limited environment.[33–36] However, these alterations in mitochondrial function can have deleterious effects in a nutrient-sufficient environment postnatally. Previous microarray studies from e21.5 foetuses of LP-fed rat dams revealed that the cellular respiration pathway was one of the most affected by LP exposure *in utero*.[37] At this time they found genes associated with tricarboxylic acid cycle and ATP production were upregulated. However, in 3 month old LP-fed rat offspring, expression of respiratory genes encoding for malate dehydrogenase, citrate synthase, *ATP6*, and *ND4L* were downregulated.[38] Cellular respiration was also significantly affected in the LP mouse based on GO enrichment analysis with 30 transcripts encoding for electron transport chain (ETC) enzyme subunits significantly decreased in the LP group at d30. Similar observations were also found in insulin target tissues, the liver and skeletal muscle, in microarray experiments conducted in LP-fed C57Bl/6 mice at birth.[39] Genes related to oxidative phosphorylation were the most over-represented genes altered in the liver and skeletal muscle. Furthermore, measurements of oxidative phosphorylation in the liver and skeletal muscle were reduced in IUGR rats induced by bilateral uterine artery ligation, demonstrating mitochondrial dysfunction and reduced ATP production.[35,36] Therefore, not only is the pancreas impacted by the foetal environment, but programming of the mitochondria is also observed in other organs involved in glucose homeostasis.

β-Cell gene expression of antioxidants is relatively low and therefore the β-cell is vulnerable to ROS.[40,41] Mitochondrial function is particularly susceptible to oxidative damage, leading to decreased mitochondrial ATP synthesis.[42] Specifically in mouse islets, increased ROS production and mitochondrial damage resulted in decreased glucose stimulated ATP synthesis and deficiencies in insulin secretion.[43] Moreover, mitochondrial dysfunction and ROS production play a role in the development and progression of type 2 diabetes.[21,22,44] In female LP mouse offspring reduced expression of many antioxidants was observed in the whole pancreas, potentially increasing susceptibility to ROS. Islets of IUGR rats were shown to have a gradual increase in ROS production, oxidative stress, and impaired ATP production with age.[45] Mitochondrial dysfunction resulted in impaired insulin secretion from isolated islets of IUGR rats.

Collectively, our data suggest that the LP diet has a significant impact on genes coding for oxidative phosphorylation, antioxidant defence, and islet function. Previous observations in LP mice showed no changes in fasting blood glucose levels, serum insulin concentration, pancreatic insulin content, or apoptosis in neonates.[3] However, early postnatal changes in gene expression may underlie the later development of β-cell dysfunction.

Additionally, programming of the pancreas by LP during foetal life reduces β-cell plasticity. LP offspring treated with multiple low doses of STZ were unable to regenerate and restore β-cell mass to the level of sham-injected mice. In contrast, regeneration of β-cell mass was robust in C-fed offspring. In the current study we mined microarray data for genes that may be altered by LP exposure resulting in an inability to regenerate β-cell mass.

As we have examined the whole pancreas, consideration was given to the exocrine compartment. While some functional deficits within the exocrine pancreas are associated with STZ,[46] it is thought to be the result of the diabetic condition. We have used modest doses of STZ that do not yield overt diabetes, but a transient hyperglycemia, which is corrected within four weeks of treatment.[3] No relative change in weight gain was observed in STZ-treated versus control mice over the period of study, therefore it seems unlikely that the changes in gene expression observed in whole pancreas are biased substantially by the exocrine tissue.

The important β-cell transcription factor, Pdx1 was decreased in STZ and LP groups, while Nkx2.2 was decreased in LP + STZ mice relative to C-fed mice. Nkx2.2 is important for islet development and the maintenance of mature β-cell identity, and regulates mature β-cell function.[47–49] Pdx1 is upregulated during β-cell regeneration.[50,51] Reduced expression of Pdx1 and Nkx2.2 may contribute to reduced β-cell regenerative capacity following STZ.

Other genes potentially key to β-cell regeneration include members of the *Reg* gene family. Upregulation of Reg1, Reg2, and Reg3δ (mouse INGAP) at the gene or protein level correlated with β-cell regeneration and diabetes reversal in several models.[25,52–54] Administration of recombinant REG1 to 90% pancreatectomized rats ameliorated diabetes, with increased β-cell mass.[55]

Further support for the *Reg* genes as facilitators of β-cell regeneration was observed from two microarray studies. The first analysed pancreatic RNA samples from 3 month-old mice treated with multiple doses of STZ (80 mg/kg).[56] Fifteen days after STZ treatment, gene expression for *Reg2*, *Reg3α*, *Reg3β*, *Reg3γ*, and *Reg3δ* were all increased. Additionally, *Reg1* expression was significantly upregulated when quantified by northern blot hybridization. A second study examined gene profiles in 2 month-old Balb/c mice following 50% pancreatectomy.[57] Twenty-four hours after surgery, an upregulation of *Reg1*, *Reg2*, *Reg3γ*, and *Reg3δ* was noted. In the current study, our microarray analysis did not suggest any significant differences in *Reg* gene expression in the C + STZ group versus C-fed mice. The disparity between these studies could be related to the age of the mice (2-3 months versus 30 days) and the insult used (50% PPx or 80 mg/kg versus 35 mg/kg STZ). However, we did observe a significant increase in gene expression by qPCR for *Reg1* in STZ treated mice at d7 in both whole pancreas and isolated islets.

Despite a significant increase in *Reg1* mRNA in the LP + STZ group, examination of protein presence suggested

a reduction compared to the C + STZ group. A few possibilities may explain this discrepancy. First, increased protein ubiquitination may target REG1 for proteasomal degradation or other post-translational modification leading to protein instability. Some proteins, such as β-catenin and cyclins, are regulated via the ubiquitin-proteasomal pathway,[58,59] however, it is not known if REG1 is post-translationally modified under normal circumstances. These changes could represent a general effect of LP exposure, affecting many proteins and therefore cellular function. Secondly, an increased rate of secretion of REG1 in an effort to stimulate β-cell regeneration could impact the measurement of REG1 islet content.

REG1 is mitogenic to β-cells[55,60,61] and may serve to rescue regeneration in LP + STZ mice. We examined the proliferative capacity of isolated islets in response to exogenous REG1α treatment. REG1α stimulated a doubling of the β-cell proliferation rate in C + sham islets. Proliferation was elevated in untreated C + STZ islets at 2.8% of insulin$^+$ cells compared to untreated control islets (1.2%), suggesting that STZ alone resulted in an increase in β-cell proliferation. This is consistent with a β-cell proliferation rate of 2.5% from PCNA stained pancreatic sections of C + STZ mice at d7 (Cox A.R., unpublished observations). The lack of mitogenic effect of REG1α on C + STZ islets may suggest a delayed upregulation of Reg1 receptor expression on the β-cell surface, or be related to the increased expression of endogenous Reg1 observed here. A failure of exogenous REG1 to further increase β-cell proliferation could suggest that the intracellular signalling pathway was saturated, reinforcing a key role of REG1 in the regenerative pathway.

Proliferation of β-cells in LP and LP + STZ islets was low and was not increased further with REG1α, consistent with our hypothesis that foetal LP programming may alter responsiveness of β-cells to mitogenic stimuli during regeneration. This could reflect reduced expression of the Reg1 receptor, exostosin-like 3 (EXTL3) under LP conditions. More probable, the downstream signalling pathway is differentially regulated in LP offspring limiting Reg1 induced proliferation. The JNK and MAPK pathways have been implicated in Reg1 signalling, with activation of Cyclin D1 expression inducing cell growth.[62,63] The MAPK phosphatase, MKP-1, was shown to inhibit JNK activity, potentially acting as a negative regulator or feedback mechanism of REG1 stimulated growth.[63] Increased MKP-1 activity or other regulators of the MAPK growth signalling pathways in LP offspring may limit REG1 induced β-cell proliferation.

These studies have significant implications for humans with respect to disease development and treatment. Here we have demonstrated that foetal nutrition alters programming of β-cell plasticity. β-cell expansion is important under normal physiologic conditions such as pregnancy and obesity. Altered REG1 signalling, and possibly other pathways involved in plasticity, may compromise β-cell expansion under certain physiologic conditions leading to diabetes. Moreover, not only does foetal LP exposure increase risk for glucose intolerance and diabetes, but also strategic attempts to expand β-cells in individuals suffering from diabetes would be limited.

In conclusion, these results support the role of REG1 in β-cell regeneration in C-fed offspring. However, in LP-fed offspring REG1 levels were reduced and isolated islets were unresponsive to exogenous REG1α treatment. Therefore, early nutritional insults may program the Reg1 pathway resulting in a limited ability to increase β-cell mass during metabolic stress. Furthermore, the effects of foetal protein restriction may program early gene expression necessary for many cell functions, such as oxidative phosphorylation and free radical scavenging. These alterations may critically impact β-cell function leading to the development of glucose intolerance in adulthood. Thus the previous dietary environment may predispose the offspring to adult disease through early changes in gene expression prior to changes in function.

Author contributions: All authors were involved in experimental design and manuscript review. ARC, CAB, and DEC conducted the experiments. CAB and DEC provided technical support. ARC, DC, EJA, and DJH were involved in data analysis and interpretation. ARC and DJH wrote the manuscript.

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