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Intrauterine programming of fetal islet gene expression in rats—effects of maternal protein restriction during gestation revealed by proteome analysis

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Abstract

Aims/hypothesis. Fetal undernutrition can result in intrauterine growth restriction and increased incidence of Type 2 diabetes mellitus. Intrauterine malnutrition in form of an isocaloric low-protein diet given to female rats throughout gestation decreases islet-cell proliferation, islet size and pancreatic insulin content, while increasing the apoptotic rate and sensitivity to nitrogen oxide and interleukin-1β. Hence, the influence of a low-protein diet on the development of beta-cells and islets could also be of interest for the pathogenesis of Type 1 and Type 2 diabetes mellitus. We hypothesise that the effects of a low-protein diet in utero are caused by intrauterine programming of beta-cell gene expression.
Methods. Pregnant Wistar rats were fed a low-protein diet (8% protein) or a control diet (20% protein) throughout gestation. At day 21.5 of gestation fetal pancreata were removed, digested and cultured for 7 days. Neoformed islets were collected and analysed by proteome analysis comprising 2-dimensional gel electrophoresis and mass spectrometry.
Results. A total of 2810 different protein spots were identified, 70 of which were changed due to the low-protein diet. From 45 of the changed protein spots, identification was obtained by mass spectrometry (64% success rate). Proteins induced by the low-protein diet were grouped according to their biological functions, e.g. cell cycle and differentiation, protein synthesis and chaperoning.
Conclusions/interpretation. Our study offers a possible explanation of the alterations induced by a low-protein diet in islets. It shows that in Wistar rats the intrauterine milieu could program islet gene expression in ways unfavourable for the future of the progeny. This could be important for our understanding of the development of Type 1 and Type 2 diabetes mellitus. [Diabetologia (2003) 46:1497–1511]

Keywords Proteome analysis, 2-dimensional gel electrophoresis, mass spectrometry, intrauterine malnutrition, low-protein diet, islet, diabetes, pathogenesis.

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Abbreviations: BB-DP, diabetes prone Bio Breeding; BiP, immunoglobulin binding protein; CAP-2, cyclase-associated protein 2; Cdk2, cyclin-dependent kinase 2; EC, enzyme classification; ER, endoplasmic reticulum; EST, expressed sequence tag; FBP, fuse binding protein; FBS, fetal bovine serum; GRP, glucose-regulated protein; HBSS, Hanks' balanced salt solution; HSP, heat shock protein; hnRNP, heterogenous nuclear ribonucleoproteins; IEF, isoelectric focusing; IL-1β, interleukin 1β; IGFBP, insulin-like growth factor binding protein; IP3R, inositol triphosphate 3 receptor; MALDI, matrix-assisted laser desorption/ionisation; NEK2, NIMA-related kinase 2; NEPHGE, non-equilibrium pH-gradient electrophoresis; %IOD, Percentage integrated optical density; PPK, protein phosphor kinase; Q-TOF, quadropole time-of-flight; ROS, reactive oxygen species; RPMI 1640, Roswell Park Memorial Institute 1640; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; TOAD-64, dihydropyrimidinase related protein-2; TOF, time-of-flight; 2-DG, 2-dimensional gel; 2-DGE, two-dimensional gel electrophoresis; STAT5, signal transducer and activator of transcription 5; TCP-1, T-complex protein-1 theta subunit; VEGF, vascular endothelial growth factor; WF, Wistar Furth; pI, isoelectric point.

Fetal undernutrition can result in intrauterine growth restriction and increased incidence of diseases such as Type 2 diabetes mellitus and coronary heart diseases in adult life in humans [1, 2, 3] as well as in rodents [4]. When a low-protein diet is given to female rats throughout gestation, changes such as decreased islet-cell proliferation, islet size, pancreatic insulin content and islet vascularisation are seen in the growth-restricted offspring at birth, despite normal concentrations of glucose and insulin in the maternal and fetal plasma [5]. The rate of apoptosis is increased in these islets [6]. The function of the fetal endocrine pancreas is also influenced, since islet-like structures (called islets below) neoformed in vitro after 7 days of culture, secrete less insulin in response to various secretagogues [7, 8]. Such islets are more sensitive to nitrogen oxide and interleukin-1β (IL-1β) [9]. The functional changes induced by the low-protein diet in utero are persistent, and adult offspring are insulinopenic and glucose intolerant [7].

The islets also show increased susceptibility to cytokines despite a normal diet after birth or after weaning [10]. In the pathogenesis of Type 1 diabetes it is possible that the cytokine IL-1β can play a role during the destruction of beta-cells, and it is generally accepted that the destruction of the beta cells results from interactions between environmental factors and immune mechanisms in genetically susceptible people [11]. It is important to keep interactions between genetic and environmental factors in mind when evaluating the possible role of a certain environmental factor in the cause of diabetes [12].

The influence of the low-protein diet on the intrauterine environment and development of beta cells and islets could therefore be of interest for the pathogenesis of Type 1 and Type 2 diabetes. We hypothesise, that the effects of the low-protein diet on beta-cell development and function in utero, are caused by intrauterine programming of beta-cell gene expression, effects that persist throughout life. To better understand the effect of the low-protein diet on fetal islets we used proteome analysis [13]. This method has been used successfully on the islets of neonatal Wistar Furth (WF) rats exposed to IL-1β and on the islets of diabetes prone Bio Breeding (BB-DP) rats [14, 15, 16, 17].

Our study compared the protein expression pattern of low-protein-diet fetal islets with control fetal islets. The aim was to identify proteins that change expression and discuss such changes with regard to the pathogenesis of Type 1 and Type 2 diabetes mellitus.

Materials and methods

Study design. The proteins of metabolically labelled neoformed fetal islets from fetuses (gestation day 21.5) of Wistar rats, which had been given either a low-protein diet (8% protein content) or a control diet (20% protein content) during pregnancy, were separated by 2-dimensional gel electrophoresis (2-DGE; *n*=6 for each group) on analytical gels each made of 250 fetal islets. The protein patterns from the two groups were compared and spots that changed expression were cut out of preparative gels made of approximately 200,000 Wistar islets, enzymatically digested and subjected to mass spectrometry for identification.

Animals. Adult 3-month-old, virgin, out-bred female Wistar rats (*n*=18 for each group, obtained from animal facilities at the Catholic University of Louvain, Louvain-la-Neuve, Belgium) were caged overnight with males and copulation was verified the next morning by detection of a vaginal plug. The animals were maintained at 25°C with a 10-h darkness and 14-h light cycle. One group of pregnant rats was fed a control diet (20% protein) and a second group received an isocaloric low-protein diet (8% protein). The source of protein was casein supplemented with 0.2% methionine for the control diet and with 0.08% methionine for the low-protein diet. The composition of these diets was as described [5]. Diets were purchased from Hope Farms (Woerden, the Netherlands). The diets were similar in fat content and were rendered isocaloric by adding carbohydrates to the low-protein diet. The rats were given free access to their respective diets and tap water. The low-protein-diet rats ate almost the same quantity of food, reducing weight gain during pregnancy by 10%. No difference in litter size was observed [5]. After day 21.5 of gestation, the dams were decapitated.

Pregnant inbred WF rats (*n*=90 rats, M&B, Ll Skensved, Denmark) were given standard rat chow, Altromin 1320 (Chr. Petersen, Ringsted, Denmark) and free access to tap water. Four- to five-day-old rats were used for islet isolation for preparative gels.

Ideally, neoformed fetal islets should be used for preparative gels. However, this is not possible due to the number of islets needed for preparative gels (200,000 islets) and the fact that only approximately 250 fetal islets can be taken from each rat litter. Because of the price and higher number of islets isolated per animal (200 to 300 islets per rat), neonatal WF rat islets were used for preparative gels. In vitro comparisons of susceptibility to IL-1β toxicity have previously shown that functionally islets from out-bred Wistar rats behave identically to islets isolated from the inbred sub-strain (data not shown). The overall protein expression patterns were identical in analytical and preparative gels by visual analysis, and all protein spots changing their level of expression in the low-protein-diet analytical gels were found in the WF-islet preparative two-dimensional gels (2-DG). Notably, no protein spots were expressed de novo in the low-protein-diet islets. Hence, preparative gels from WF-rat islets are suitable for mass spectrometry identification of proteins in the spots that changed levels of expression in low-protein islets.

All animal experiments were carried out according to national and international law and ethical standards. The experiments were approved by the Danish Council for Animal Welfare under the Ministry of Justice and with approval of The Animal Ethics Committees of the Catholic University of Louvain, Louvain-la-Neuve, Belgium.

Isolation, culture and labelling of islets for analytical gels. The pancreata were removed aseptically from 21.5-day-old control and low-protein-diet fetuses and placed in sterile cold Roswell Park Memorial Institute 1640 (RPMI 1640) added 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/l streptomycin and 20 mmol/l HEPES buffer (medium; Gibco, Paisley, Scotland). They were then minced and transferred to a sterile

tube containing 2 ml of medium with collagenase (specific activity 381 U/ml, Sigma, St. Louis, Mo., USA). The tube was shaken by hand for 7 to 8 min at 37°C. The digested tissue was then washed twice with cold medium. Next, the resulting cell pellets were suspended in 20 ml of medium and gently stirred at room temperature for 60 min. After centrifugation (180×*g* for 10 min), the pellets were resuspended in medium (one pancreas per 2 ml RPMI 1640+10% FBS). This suspension was distributed into Petri dishes (2 ml per dish) and incubated for up to 7 days at 37°C in a 5% CO_2 humidified atmosphere. The culture technique was slightly modified from that previously described [18]. From day 2 of culture, the medium was replaced every 24 h. These cultures provided islets that aggregated progressively on the layer of non-endocrine cells. At least three pregnant rats per culture were used giving about 30 fetuses. At the end of the culture period, three to four batches of 250 islets were collected for proteome analysis. Six different cultures were used for each experimental group. Batches of 250 neoformed islets were hand-picked and labelled with [^{35}S]-methionine (SJ 204, specific activity >37 TBq/mmol/l, containing 0.1% 2-mercaptoethanol, Amersham International, Amersham, UK). After labelling, the islets were washed three times in Hanks' balanced salt solution (HBSS; Gibco, Paisley, Scotland), the supernatant was removed and 120 µl lysis buffer (8.5 mol/l urea; ultra pure, Schwarz/Mann, Cambridge, Mass. USA), 2% Nonidet P-40 (BDH, Poole, UK), 5% 2-mercaptoethanol (Sigma) and 2% ampholytes, pH range 7 to 9 (Amersham Biotech, Upsala, Sweden) were added and the mixture was immediately frozen at −80°C.

Isolation, culture and labelling of islets for preparative gels. The amount of protein in each of the spots from the analytical gels (250 islets per gel) is very small. To ensure that spots contained enough material for mass spectrometry identification, preparative gels (20,000 islets per gel) were prepared and run.

Islets were isolated by collagenase digestion of the pancreata from 4- to 5-day-old WF rats [19]. This was followed by 4 days of preculture in RPMI 1640 added 10% FBS and incubation for 24 h in RPMI 1640 added 0.5% normal human serum (Sigma). Incubation took place in humidified atmospheric air at 37°C. The amounts of RPMI used were: 300 µl (200 islets for labelling of tracer islets to be mixed with unlabelled islets) or 3000 µl (20 000 islets for unlabelled islets). Next, islets were washed twice in HBSS and tracer islets were labelled for 4 h at 37°C in 200 µl home-made methionine-free DMEM (Gibco, Paisley, Scotland) with 10% dialysed normal human serum and 7.4 MBq [^{35}S]-methionine. The unlabelled islets for the preparative gels were incubated in the same way in 3000 µl methionine containing DMEM without [^{35}S]-methionine. To eliminate 2-mercaptoethanol, [^{35}S]-methionine was freeze-dried for at least 4 h before labelling. After labelling, the islets were washed three times in HBSS, the supernatant was removed and islets were immediately frozen at −80°C. Unlabelled islets for preparative gels were washed twice in HBSS and snap-frozen. For localisation of the spots, radioactively labelled tracer islets were mixed with the non-labelled islets and lysis buffer was added to the mixture before further preparation.

Sample preparation. The frozen islets were resuspended in 100 µl DNAse1 and RNAse A solution (Worthington, Freehold, N.J., USA) by shaking in lysis buffer for a minimum of 4 h. They were then frozen and thawed in the solution three times, stirred on ice for 20 min and freeze-dried overnight.

Measurement of [35S]-methionine incorporation. The amount of [^{35}S]-methionine incorporated was quantified by adding 10 µl FBS (0.2 µg/ml H_2O) as a protein-carrier to 5 µl of a 1:10 dilution of each sample in duplicate, followed by 0.5 ml of 10% trichloracetic acid (Merck, Darmstadt, Germany). This was left to precipitate for 30 min at 4°C before being filtered through 0.25 µm hydroxyappatite-WP filters (Millipore, Boston, Mass., USA). The filters were dried and placed in scintillation liquid (Filter-count, Canberra Packard, Zürich, Switzerland) and samples were counted (Canberra Packard 2000 scintillation counter).

Measurement of protein concentration. The Bradford method [20] was scaled down and adapted for use by adding an amount of the lysis buffers equal to the volume of the sample to the standard curve. All samples were analysed in duplicate. The colour reagents used were from Bio-Rad, Hercules, Calif., USA.

2-DGE and preparative gels. Analytical 2-DGs were produced from 250 [^{35}S]-methionine labelled neoformed control or low-protein-diet islets. Preparative 2-DGs were produced from a pool of approximately 200 000 neonatal WF rat islets. For localisation of the spots, radioactively labelled tracer islets were mixed with the non-labelled islets.

The procedure for the production of 2-DGs has been described earlier [21, 22]. Briefly, first dimension gels contained 4% acrylamide (BioRad, Richmond, Calif., USA); 0.25% bisacrylamide (BioRad, Richmond, Calif., USA); ampholytes with pH ranges pH 5 to 7, pH 3.5 to 10, pH 7 to 9, pH 8 to 9.5 (Amersham Biotech) and pH 5 to 7 (Serva, Heidelberg, Germany); and Nonidet P-40 (BDH, Poole, UK). Equal amounts of protein (175 to 200 µg for preparative gels) and counts per minute (10^6 cpm) of each sample were applied to the gels. We made gels both for isoelectric focusing (IEF; pH 3.5 to 7) and for non-equilibrium pH-gradient electrophoresis (NEPHGE; pH 6.5 to 10.5). Second dimension gels contained 12.5% acrylamide and 0.063% bisacrylamide and were run overnight. After electrophoresis, the gels were fixed and treated with Amplify (Amersham International) before being dried. The gels were exposed to a PhosphoImager screen and the protein-pattern was scanned on a PhosphorImager (ADC 270 AGFA, Kontich, Belgium). The resulting approximately 8-MB images were converted to 16-bit tiff images for image analysis.

Measurement of molecular weight and isoelectric point. The identity of landmark proteins was determined by the use of mass spectrometry. The theoretical molecular weight (M_r) and isoelectric point (pI) were calculated using the Compute pI/M_r tool at the ExPASy Molecular Biology Server (http://ca.expasy.org/) [23] and observed M_r and pI values were then calculated by linear regression with respect to the proteins' mobility. The M_r and pI for individual proteins on the gels were interpolated from the observed M_r and pI of the landmark proteins.

Computer analysis of 2-DGs and statistics. Computer analysis of the analytical gels was carried out using the BioImage program 2D-Analyzer (version 6.1; Genomic Solutions, Ann Arbor, Mich., USA) [15]. This program finds and quantifies the protein spots and matches the gels together. To obtain sufficient accuracy the quantification and matching done by the computer program have to be controlled and manually corrected. Percentage integrated optical density (%IOD) is obtained for each single spot for statistical analysis. Significant changes in %IOD between individual spots in the control and low-protein-diet groups were identified using a double-sided non-paired Students *t* test comparing six independent experiments from each group. Changes with a *p* value of less than 0.01 were considered significant. For most proteins this reflects a 1.5-fold or higher change in expression level.

Protein identification by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS). The 70 protein spots of interest were cut out of the dried gel with a scalpel. Enzymatically in-gel digestion was carried out as described [24, 25] with minor modifications [26]. The excised gel plugs were washed in digestion buffer (50 mmol/l NH_4HCO_3, pH 7.8), acetonitrile (60/40) (Sigma) and dried by vacuum centrifugation. Endoproteinase Lys-C (10 ng/µl; Calbiochem, La Jolla, Calif., USA) dissolved in 50 mmol/l NH_4HCO_3, pH 7.8, was added to the dry gel pieces in the cases where the protein in the gel was larger than 20 M_r. Modified trypsin (10 ng/µl; Promega, Madison, Wis., USA) dissolved in 50 mmol/l NH_4HCO_3, pH 7.8, was added to the dry gel pieces in the cases where the protein in the gel was smaller than 20 M_r. The solutions were incubated on ice for 1 h. After removing the supernatant, additional digestion buffer was added and digestion was continued at 37°C for 4 to 18 h. An aliquot of the supernatant from each digestion was concentrated and desalted on GELoader tip micro-columns packed with Poros R2 (PerSeptive Biosystems, Framingham, Mass., USA) and eluted directly onto the MALDI target. MALDI time-of-flight (TOF)-mass spectrometry was done using a Voyager STR (PerSeptive Biosystems) equipped with delayed extraction. All spectra were obtained in positive reflector mode using an accelerating voltage of 20 kV. MALDI TOF-mass spectrometry spectra were processed in MoverZ (Genomic Solutions) and the peak lists were searched using ProFound [27] or Mascot [28]. The protein identifications were examined according to a previous study [29]. In some cases, where the MALDI-mass spectrometry analysis gave a good spectrum but ambiguous identification, the remaining peptide mixture was concentrated and desalted and analysed by nano-electrospray tandem mass spectrometry using a Q-Tof (quadrupole time-of-flight) hybrid quadrupole/orthogonal-acceleration TOF mass spectrometer (Micromass, Manchester, UK). The fragment spectra were manually interpreted using the MassLynx software and the resulting peptide sequences were searched against protein/cDNA/expressed sequence tag databases using various search programs available on the Internet.

The criteria for identification were mainly set on the number of peptides matching the entry in the database, and the distance in matched peptides to the background of randomly scoring proteins. However, sequence coverage, alternative cleavage patterns and methionine oxidation were also taken into consideration [29]. The experimentally determined molecular weight of the proteins obtained from the 2-DGs was also evaluated for unambiguous identification.

Protein information. Information about the identified proteins' known and putative biological functions was found at The ExPASy Molecular Biology Server [30] and at The National Center for Biotechnology Information (NCBI) [31] or in textbooks of biochemistry or molecular biology.

Results

A total of 2810 different protein spots were located on the 2-DGs of proteins extracted from neoformed fetal islets from the control diet group and the low-protein-diet group, respectively. The spots were quantified according to incorporation of the radioactive isotope [^{35}S]-methionine and %IOD. Differential display between the protein expression patterns of the low-protein and control groups showed a noticeable change in the expression of 70 spots. Of these 15 were located on the basic (NEPHGE) side, whereas 55 were on the acidic side (IEF) (Fig. 1). When compared to the control group, 39 spots were up-regulated and 31 down-regulated in the low-protein-diet islets.

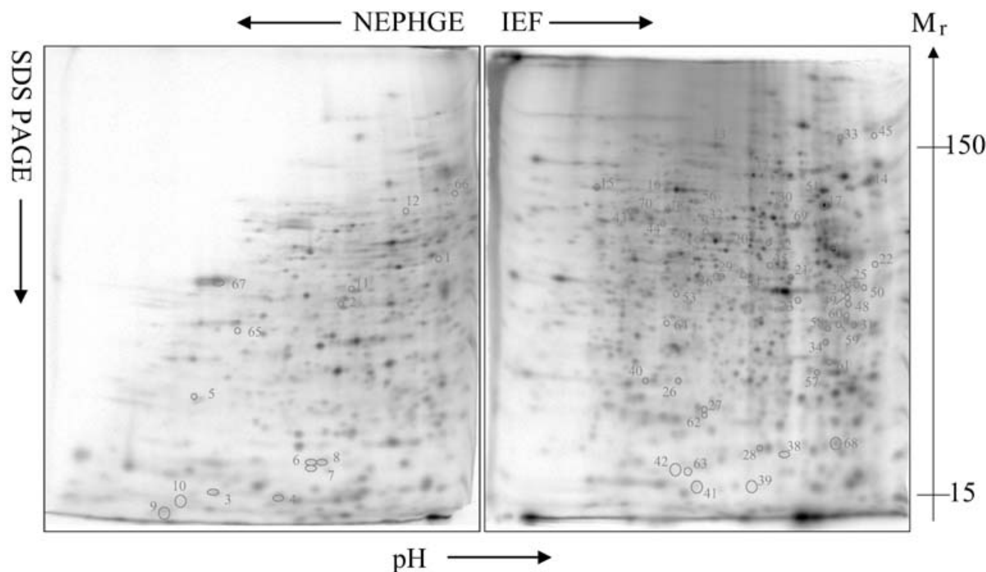

Fig. 1. Two-dimensional gels of low-protein-diet islets labelled with [^{35}S]-methionine for 4 h. The gels shown are representative low-protein-diet gels from six independent experiments. The marked protein spots are those where low-protein-diet islets have significantly changed expression in comparison to control islets. IEF gel (pH 3.5 to 7) is on the right and NEPHGE gel (pH 6.5 to 10.5) on the left. The numbers correspond to the proteins in Tables 1, 2, and spots with more than one protein are marked with the number in the figure and with the number and suffix a or b in Tables 1, 2. Theoretical pI and M_r values of proteins were calculated by the Compute pI/M_r tool at the ExPASy Molecular Biology Server on the amino acid sequence reported in the protein database only. Post-translatory modifications are not taken into account

Table 1. Identified proteins changed by a low-protein diet

Protein no.	Protein name	Accession no.	% IOD ratio	pI in gel	M_r in gel	Theo* pI	Theo* M_r	Function	References
Energy transduction and redox potentials									
1	ATP synthase alpha chain, mitochondrial	P15999	0.4	7.6	52,900	9.22	58,826	Regulatory subunit of mitochondrial ATP synthase. EC 3.6.3.14	[32]
9a	Ubiquinol-cytochrome C reductase complex 14 kDa protein	P14927	3.8	9.3	16,300	8.75	13,399	Part of mitochondrial respiratory chain. Redox-linked proton pumping. EC 1.10.2.2	[33, 34, 35]
Glycolysis and Krebs Cycle									
2	Isocitrate dehydrogenase (NADP), mitochondrial	P48735	1.6	8.5	45,000	8.88	50,948	Krebs Cycle, energy production. EC 1.1.1.42	[33, 34, 35]
40	Phosphoglycerate mutase, brain form	P18669	2.9	6.1	27,500	6.75	28,673	Glycolysis and phosphorylation. EC 5.4.2.1, EC 5.4.2.4, EC 3.1.3.13	[33, 34, 35]
RNA and DNA metabolism									
4	Histone H2B	Q00729	0.4	8.8	17,500	10.29	14,093	Part of histone octamer. Component of the transcriptional machinery	[36]
5a	Heterogeneous nuclear ribonucleoprotein A3	P51991	0.5	9.28	36,500	8.74	39,686	Ribonucleosomes, mRNA preparation, splicing and export	[37, 38]
21	Heterogeneous nuclear ribonucleoprotein F	P52597	0.8	5.08	49,000	5.38	45,672	Ribonucleosomes, mRNA preparation, splicing and export. Preference for guanosine-rich sequences	[37, 38, 39]
43b	GMP synthase	P49915	1.4	6.4	77,300	6.42	76,751	De novo synthesis of guanine. Down-regulated when cell proliferation is inhibited. EC 6.3.5.2	[40]
41	Nucleoside diphosphate kinase A	Q05982	0.7	6.1	18,100	5.96	17,193	Nucleoside triphosphate synthesis. EC 2.7.4.6	[33, 34, 35]
57	Adenylate kinase isoenzyme 4, mitochondrial	Q9WUS0	0.5	4.8	28,000	7.80	25,203	Nucleotide homeostasis and production. EC 2.7.4.3	[33, 34, 35]
Protein synthesis and metabolism									
5b	Ribosomal protein S4	Q9CXNO	0.5	9.20	36,500	10.16	29,615	Part of ribosomal complex. Involved in protein synthesis	[33, 34, 35]
64	60S acidic ribosomal protein P0	P19945	0.13	6.0	34,600	5.91	34,215	Part of ribosomal complex. Involved in protein synthesis, interacts with elongation factor 1 and 2	[41, 42]
68	60S Ribosomal protein L11	Q29205	2.0	4.40	20,900	9.97	18,818	Part of ribosomal complex. Involved in protein synthesis	[33, 34, 35]
67	Elongation factor 1-alpha 1	P20001	0.6	8.90	52,500	9.10	50,114	Transfers the next amino acid to the peptide being produced during protein synthesis	[33, 34, 35]
56	Glycyl-tRNA synthetase	Q04451	0.7	5.8	86,000	6.24	81,878	Activation of amino acids for translation linking it to tRNA before taken up by elongation factor 1-alpha 1. EC 6.1.1.14	[33, 34, 35]

Table 1. (continued)

Protein no.	Protein name	Accession no.	% IOD ratio	pI in gel	M_r in gel	Theo* pI	Theo* M_r	Function	References
23	Carboxy-peptidase B	P15086	0.3	4.90	42,600	6.29	47,367	Cleavage of polypeptides to minor peptides or amino acids. EC 3.4.17.2	[33, 34, 35]
26a	Proteasome subunit beta type 7	Q9JHW0	0.5	5.9	27,900	8.14	29,927	ATP-dependent degradation of ubiquitin-marked proteins. EC 3.4.25.1	[43]
53	Isovaleryl-CoA dehydrogenase, mitochondrial	P12007	0.3	6.0	41,700	8.03	46,435	Leucine catabolism. EC 1.3.99.10	[33, 34, 35]
Protein folding and chaperones									
17	78 kDa glucose-regulated protein (HSP70 family), immunoglobulin binding protein (BiP)	P06761	1.2	4.8	92,200	5.07	72,347	Folding and assembly of secreted and membrane-bound proteins in endoplasmatic reticulum	[44, 45]
18a	Heat shock protein 75 kDa, mitochondrial, tumour necrosis factor type 1 receptor associated protein (HSP75)	Q9CQN1	1.8	6.1	72,200	6.25	80,209	Chaperone. Involved in cytotoxicity and induction of protective activity by refolding of proteins	[46, 47, 48]
44	T-complex protein 1, gamma subunit	P80318	2.5	6.1	66,300	6.28	60,630	Chaperoning and folding of actin and tubulin	[49, 50]
20a	T-complex protein 1, theta subunit	P50990	1.6	5.4	61,400	5.42	59,621	Chaperoning and folding of actin and tubulin	[49, 50]
52	T-complex protein 1, theta subunit	P50990	0.6	5.3	61,100	5.42	59,621	Chaperoning and folding of actin and tubulin	[49, 50]
20b	Protein disulfide isomerase A3 (calreticulin)	P11598	1.6	5.4	61,400	5.88	56,623	Folding and assembly of secreted and membrane-bound proteins in endo-plasmatic reticulum. EC 5.3.4.1	[51]
55a	Protein disulfide isomerase A3 (calreticulin)	P11598	0.5	6.0	61,800	5.88	56,623	Folding and assembly of secreted and membrane-bound proteins in endoplasmatic reticulum. EC 5.3.4.1	[51]
22	Endo plasmin, protein kinase 98, (PPK98), (HSP 90 family)	Q29092	0.3	4.2	64,500	4.75	92,471	Chaperone preventing protein aggregation. Organisation and maintenance of cyto-architecture	[52]
51	Endo plasmin, protein kinase 98, (PPK98), (HSP 90 family)	Q29092	1.5	5.0	98,700	4.75	92,471	Chaperone preventing protein aggregation. Organisation and maintenance of cyto-architecture	[52]
69	Hsc70-ps1 gene	P08109	3.0	5.0	63,700	5.37	70,871	Chaperone guiding folding, assembly, intracellular transport, refolding and degradation of proteins. Functions together with HSP90 family	[52, 53, 54]

Table 1. (continued)

Protein no.	Protein name	Accession no.	% IOD ratio	pI in gel	M_r in gel	Theo* pI	Theo* M_r	Function	References
Cell-cycle, differentiation, signal transduction and transcription									
18b	Serotransferrin	Q29443	1.8	6.1	72,200	6.75	77,753	Iron transport. Important for cell proliferation	[55]
14	Lactotransferrin	Q9TUM0	1.7	4.3	119,800	8.66	77,211	Iron transport. Immune modulatory properties. Regulates G1 cyclins and induces growth arrest. Activates IL-1β transcription	[56, 57, 58]
25	G1/S-specific cyclin D2	P30280	0.5	4.4	53,100	5.04	32,897	Cell-cycle regulation. Promotes progression through G1 phase in cell division	[59, 60]
29	Adenylyl cyclase-associated protein 2	P40123	1.6	5.6	47,200	5.95	52,824	Interaction with actin. Inhibition of cell growth	[61, 62, 63]
46	Nucleobindin 2	Q9JI85	0.6	4.7	57,000	5.02	50,090	Calcium-binding protein. Promotes anti-DNA autoimmune response. Calcium homeostasis	[64, 65, 66, 67, 68]
55b	Dihydropyrimidinase related protein-2 (TOAD-64)	P47942	0.5	5.9	61,800	5.95	62,278	Neurogenesis and cellular differentiation	[69, 70]
58	Small glutamine-rich tetratricopeptide repeat-containing protein	O70593	0.5	4.6	35,500	5.05	34,157	Cell-cycle control, transcription repression, protein kinase inhibition, stress response, protein transport and cyclin degradation	[71, 72]
61a	Annexin V	P14668	0.5	4.6	30,200	4.93	35,613	Secretory cell functions. Protein kinase C inhibition. Signal transduction	[73, 74]
62	Fertility protein SP22 (CAP1 protein)	O88767	0.5	5.7	24,000	6.32	19,974	Cell cycle, Ras-dependent signal transduction	[75, 76, 77]
66	Fuse Binding Protein 2	Q92945	0.6	6.1	72,000	8.52	68,436	Transcription factor. Regulator of c-myc expression. Cell cycle, growth, proliferation, differentiation and apoptosis. Inhibition of Fuse Binding protein 2 arrests proliferation	[78, 79]
63	Low molecular weight cytosolic acid phosphatase, acid phosphatase 1 (APC1)	P41498	0.4	5.8	21,300	6.10	18,195	Phosphatase involved in control of cell growth, proliferation and differentiation. APC1-gene polymorphisms favour body mass increase and lipid levels	[80, 81]
Cellular structure									
43a	WD-repeat protein 1	O88342	1.4	6.4	77,300	6.11	66,406	Binds β-actin. Cytoskeleton. WD proteins are involved in signal transduction and cell-cycle control	[82, 83]
70	WD-repeat protein 1	O88342	1.9	6.3	76,200	6.11	66,406	Binds β-actin. Cytoskeleton. WD proteins are involved in signal transduction and cell-cycle control	[82, 83]

Table 1. (continued)

Protein no.	Protein name	Accession no.	% IOD ratio	pI in gel	M_r in gel	Theo* pI	Theo* M_r	Function	References
47a	Vimentin	P31000	0.5	4.5	50,200	5.06	53,602	Cellular structure. Support cellular membranes and keep nucleus and other organelles in a defined place within the cell	[33, 34, 35]
50	Vimentin	P31000	0.5	4.3	52,900	5.06	53,602	Cellular structure. Support cellular membranes and keep nucleus and other organelles in a defined place within the cell	[33, 34, 35]
48	Cytokeratin 21, rat keratin, type 1, cytoskeletal 21	P25030	0.5	4.5	44,700	5.28	49,256	Cellular structure and cyto-skeleton	[33, 34, 35]
49	Cytokeratin 21, rat keratin, type 1, cytoskeletal 21	P25030	0.3	4.5	45,900	5.28	49,256	Cellular structure and cyto-skeleton	[33, 34, 35]
Cellular defence									
27a	Antioxidant protein 2	O35244	0.8	5.7	24,800	5.65	24,687	Protection against reactive oxygen species (ROS). Signal transduction and phospholipase activity. EC 3.1.1.- and EC 1.11.1.7	[84, 85]
42	Calgranulin B	P31725	0.4	6.0	18,400	6.72	12,918	Chemo-attraction and defense. Calcium-binding protein. Increased in patients with inflammatory diseases	[86, 87]
Miscellaneous functions									
3	Alpha-S1-casein	P02662	0.5	9.0	18,300	4.98	24,529	Transport of calcium phosphate	[33, 34, 35]
32	Serum albumin	P02769	1.7	5.8	74,200	5.82	69,293	Homeostasis	[33, 34, 35]
27b	Synaptosomal-associated protein 29	O95721	0.8	5.7	24,800	5.56	28,970	Regulator of membrane fusion trafficking, intracellular transport	[88, 89]
38	Glucagon	P06883	0.5	5.0	19,900	5.42	20,846	Glucose homeostasis	[33, 34, 35]

The protein numbers correspond to the number of the protein spots in Fig. 1. Protein names and accession number refer to the Swiss-Prot and TrEMBL databases [30]. %IOD ratios are the ratio of integrated optical density between low-protein -diet and control gels. Values below 1 indicate down-regulated and values above 1 up-regulated proteins in low-protein diet islets. The proteins are ordered according to functional groups. The M_r and pI are given as values obtained directly by the gel analysis and as the theoretical M_r and pI calculated from the amino acid sequence. In some spots more than 1 protein was identified. These proteins are marked with an "a" or "b" for the same match number. Proteins mentioned more than once are found in more than one spot. Specific functions of the proteins are mentioned in the function column. EC numbers mentioned under function refer to The International Union of Biochemistry and Molecular Biology nomenclature of enzymes [90]. Relevant references are noted for each protein
* Theoretical

The 70 differently expressed spots were excised from preparative gels and the proteins were identified by MALDI-mass spectrometry peptide mass mapping. In the majority of the spots where the theoretical M_r or pI of the protein did not match the M_r and pI measured by us, the deviation could be accounted for by post-translational modifications of those proteins [e.g. transit peptides (protein numbers 1, 2, 53), signal peptides (protein number 23), glycosylation (protein numbers 14, 46), phosphorylations (protein numbers 17, 57, 68), pre-forms of the mature proteins or degradation products]. Of the 70 spots excised, 45 resulted in successful protein identification, yielding 45 different proteins (64% success rate) (Table 1).

In six spots two proteins were identified on the basis of the MALDI-mass spectrometry peptide mass

Table 2. Spots with unidentified proteins which significantly changed expression

Spots with unidentified proteins

Protein no.	MALDI result	%IOD ratio	pI in gel	M_r in gel
6	Weak MALDI map—no match	1.9	8.6	22,500
7	Weak MALDI map—no match	2.2	8.6	21,600
8	Weak MALDI map—no match	2.3	8.6	22,600
9b	Good MALDI map—unknown	3.8	9.3	16,300
10	Weak MALDI map—no match	0.6	9.2	17,100
11	Good MALDI map—unknown	1.4	8.4	47,800
12	Very few peptides, no ID	0.4	7.2	68,100
13	No MALDI map	2.0	5.7	154,600
15	Very few peptides, no ID	1.7	6.7	103,700
16	Very few peptides, no ID	2.0	6.1	93,700
19	Good MALDI map—unknown	2.0	5.7	64,200
24	Good MALDI map—unknown	0.7	4.5	48,700
26b	Good MALDI map—unknown	0.5	5.9	27,800
28	Weak MALDI map—no match	2.0	5.4	20,600
30	No MALDI map	0.7	5.2	75,000
31	Good MALDI map—unknown	1.5	4.4	39,400
33	Weak MALDI map—unknown	1.7	4.1	177,200
34	Weak MALDI map—unknown	0.7	4.7	34,300
35	Good MALDI map—unknown	2.3	5.3	52,900
36	No MALDI map	2.3	5.6	47,300
37	No useful MALDI map	1.7	5.4	110,700
39	Good MALDI map—unknown	0.5	5.4	17,900
45	No MALDI map	0.5	3.8	177,300
47b	Good MALDI map—unknown	0.5	4.5	50,200
54	No MALDI map	3.2	5.4	48,600
59	No MALDI map	0.6	4.6	36,600
60	Good MALDI map—unknown	0.4	4.5	40,400
61b	Good MALDI map—unknown	0.5	4.6	30,200
65	No MALDI map	2.0	9.0	44,700

Protein numbers are arbitrary numbers and correspond to the numbers of the spots on the gel image in Fig. 1. The %IOD ratio is the ratio of integrated optical density between gels compared. Numbers below 1 are down-regulated and numbers above 1 are up-regulated proteins in low-protein-diet islets. The molecular weigh in M_r and pI are the values obtained directly from the gels. In some spots more than one protein was identified. These proteins are marked with a "b". The identified protein for the same spot can be found in Table 1 and is marked with an "a" and the same protein number

map and subtractive database searching [29]. In addition, in four spots one protein was identified, the presence of a large number of unassigned peptides in these spots indicated clearly that more than one protein was present within these spots. A number of other spots yielded good-quality MALDI-mass spectrometry peptide mass maps, resulting in ambiguous protein identifications (Table 2).

In total, useful MALDI-mass spectrometry peptide mass maps were obtained from 61 of the 70 spots analysed (87%). The identified proteins were categorised into functional subgroups (Table 1) and discussed in relation to known changes in protein expression in low-protein islet function.

The proteins behind the significantly changed spots are from the following pathways and functional groups: (i) energy transduction, redox potentials; (ii) glycolysis and Krebs cycle; (iii) RNA and DNA metabolism; (iv) protein synthesis and metabolism; (v) protein folding and chaperoning; (vi) cell cycle, differentiation, signal transduction and transcription; (vii) cellular structure; (viii) cellular defence; and (ix) miscellaneous (Table 1).

Discussion

Proteome analysis comprising 2-DGE, mass spectrometry and bio-informatics allows identification of different pathways and proteins responsible for the functional changes observed in the low-protein-diet islets. Proteome analysis was previously done on islets from 4-day-old rats exposed to IL-1β in vitro and showed that 4 to 6% of the protein spots were significantly changed by the cytokine treatment [15, 16, 17, 91].

Our study showed that changes in protein expression pattern occur in fetal rat islets after protein restriction during gestation. A success rate of 64% in identifying significantly changed proteins in low-pro-

tein-diet fetal islets compared to control islets is comparable with previous findings [16, 17]. Overall, the specific protein expression changes were compatible with the functional findings reported for this model.

Admittedly, the control and low-protein-diet islets resulted from a process of neoformation in vitro, comprising about 90% beta cells, and for 7 days the cells proliferated and differentiated in culture, during which they were withdrawn from the maternal milieu. However, control and low-protein-diet islets were kept under the same environmental influences while in vitro. So the difference in the phenotype seen after 7 days in endocrine cells withdrawn from the abnormal metabolic milieu demonstrated maternal programming. Such programming was not unexpected because of the lasting alteration in insulin secretion in adult offspring [92] and the increased vulnerability to cytokines [10]. Still, this study shows that changes in the islet protein expression pattern are acquired.

We are unable to point out if the specific protein expression alteration resulted from reduced protein intake or increased sugar intake. However, although the total quantity of amino acids was identical in the maternal and fetal plasma of both groups, plasma concentrations of certain amino acids, including taurine, were reduced in low-protein-diet fetuses [93]. We suggest that the low taurine plasma levels could be involved in these modifications, since we have previously shown that supplementation of the maternal low-protein diet with taurine restored most of the changed functional parameters at the fetal stage and at adulthood [9, 10, 94, 95].

We have chosen to study whole islets, since cells in an islet work together as an organ and separation of the different cell types would induce a cellular stress response changing the specific protein expression. Indeed, protein changes might originate from cell types other than beta cells, but still they changed expression in this model and are therefore of potential interest for proper islet function.

A brief discussion of proteins possibly and partly responsible for the known morphological and functional features of the low-protein-diet animal beta cell is given below. This study did not allow us to pinpoint a single protein or proteins responsible for the phenotypical changes induced by the low-protein-diet. Rather, it is possible that the phenotypical changes and lasting effects of the low-protein-diet are a product of relatively small expression changes in many proteins. Due to the specific experimental conditions (e.g. labelling interval and the general culture conditions), we were not able to distinguish between primary or secondary changes in protein expression.

Proteins putatively responsible for the lower beta-cell mass and proliferation of LP islets. Beta-cell mass, islet size, islet-cell proliferation and pancreatic insulin content were lower in the fetuses of dams on low-protein-diets than in those on control diets [5]. Our study showed that the expression of 11 proteins involved in cell cycle and proliferation was modified in the low-protein-diet islets, eight of these proteins being down-regulated and three up-regulated. Some of these proteins could be involved in insulin secretion.

Adenyl-cyclase associated protein 2 (cyclase-associated protein 2, CAP-2) was up-regulated in low-protein-diet islets. Perturbation of CAP levels in mammalian cells seems to influence actin dynamics and could play a role in vesicle trafficking and development [63]. Increased expression could contribute to the difficulty low-protein-diet islets have in releasing insulin by exocytosis [8]. Serotransferrin binds insulin-like growth factor binding protein (IGFBP)-3 in the fetal endocrine pancreas [6]. Although both transferrin and IGFBP-3 stimulate cell growth, their combination inhibits it [55], probably by reducing endogenous production of IGF-II [96]. Of interest, IGF-II was reduced in low-protein-diet islet cells [6]. Lactotransferrin expression was up-regulated in low-protein-diet islets. Lactotransferrin inhibits cell proliferation by inducing growth arrest at the G1 to S transition of the cell cycle through a decrease in the level of cyclin-dependent kinase 2 (Cdk2) and cyclin E [56]. We have postulated that the cell cycle is lengthened in low-protein-diet fetal and neonatal islets, because the number of islet cells positive for cyclin D1 (nuclear protein which accumulates in G1) was increased, while the number of NIMA-related kinase 2 (NEK2, a serine/treonine kinase which accumulates at the G2 to M phase transition) positive islets was decreased [6].

The following proteins involved in the cell cycle processes were all down-regulated: G1/S-specific cyclin D2, Fuse binding protein 2 (FBP), fertility protein SP22, Low molecular weight cytosolic acid phosphatase, dihydropyrimidinase-related protein-2 (TOAD-64). G1/S-specific cyclin D2 promotes progression through G1 phase [60]. Its mRNA and protein are expressed at high levels in proliferative phase endometrium [97]; therefore, it is not surprising to observe reduced expression of G1/S-specific cyclin D2 in low-protein-diet islets. This protein is also up-regulated when embryonic stem cells commit to neural differentiation [98], suggesting that low-protein-diet islet cells could be less differentiated. In addition G1/S-specific cyclin D2 is a target gene for Signal Transducer and Activator of Transcription 5 (STAT5), which activates transcription [99]. FBP 2 is a transcription factor which participates in the regulation of c-myc expression [78] and loss of FBP function arrests cellular proliferation [79]. Low molecular weight cytosolic acid phosphatase influences cell division, differentiation and growth [81]. Therefore, a reduction of its expression should lead to lower islet-cell proliferation. TOAD-64 is present in fetal islets although it is described as a marker of commitment to neuronal differentiation during fetal development [100]. In low-pro-

tein-diet islets TOAD-64 was down-regulated. Interestingly, maternal dietary choline deficiency decreases the rate of mitosis and increases the number of apoptotic cells associated with decreased expression of TOAD-64 in the progenitor neuroepithelium adjacent to the septum [100].

Decreased insulin secretion, lower IGF-I, IGF-II, vascular endothelial growth factor (VEGF) in the insular-cell and islet-cell proliferation have all been reported in fetuses from mothers fed on a low-protein diet [6, 7, 95]. In support of this, several proteins involved in DNA and RNA metabolism and protein synthesis were down-regulated. Histone H2B is part of the histone octamer, around which DNA is wrapped in the nucleosome, and functions together with chromatin as an integral component of the transcriptional machinery [36]. Together with heterogeneous nuclear ribonucleoproteins (hnRNP) A3 and F participating in post-transcriptional splicing and mRNA export [37, 38], it was down-regulated in the low-protein-diet islets. Nucleoside diphosphate kinase A and adenylate kinase isoenzyme 4 are both involved in maintaining intracellular concentrations of di-nucleotide-tri-phosphates at the expense of ATP, and were both down-regulated, indicating decreased production of nucleosides. Peptides are synthesised by stepwise elongation with amino acids in the ribosomes where glycyl-tRNA synthetase, together with elongation factor 1 alpha 1, activates and transfers the next amino acid to the peptide in an energy-requiring process. [33, 34, 35]. All these proteins were down-regulated in the low-protein-diet islets, indicating a lower production of RNA and proteins.

Proteins putatively involved in the low insulin secretion of low-protein islets. Alteration in the expression of proteins involved in cytoskeleton structure and regulation could explain not only the lower proliferation rate [6] but also the lower insulin secretion [7, 8] seen in the low-protein-diet islets. Perturbations in mammalian cells in the level of adenylyl-cyclase associated protein 2 and other members of the same group (CAPs) seem to influence actin dynamics, suggesting a fundamental role for CAPs in cytoskeleton organisation and cell growth. CAPs are thought to interact with many components of the endocytosis and vesicle trafficking regulatory pathways [63]. Annexin V could also be involved in the lower insulin secretion. It is a member of a calcium-dependent phospholipid-binding protein family, which participates in vesicular transport specifically in plasma membrane to membrane or plasma membrane to cytoskeleton interactions [74]. Likewise, nucleobindin 2 is a DNA- and calcium-binding protein, which is involved in establishing the agonist-mobilisable Golgi Ca^{2+} store, together with the sarcoplasmic and endoplasmic reticulum calcium ATPase (SERCA) and the inositol triphosphate 3 receptor (IP3R) [101]. Overexpression of this protein in the Golgi increases calcium uptake by sequestration of calcium in the Golgi lumen [101]. Being down-regulated in the low-protein-diet fetal islet, a lower intracellular calcium pool could be related to the lower insulin secretion of these islets in response to secretagogues.

Proteins involved in apoptosis rate and susceptibility to cytokines of the low-protein islets. The low-protein diet increased the rate of apoptosis and susceptibility to cytokines in the islets of the fetal progeny [9]. Ten proteins involved in protein folding and chaperoning were modified, possibly testifying to some cell stress. The endoplasmic reticulum (ER) plays several important roles in the folding, export and processing of new synthesised proteins. Various conditions can interfere with ER function and are called collectively ER stress. At least three functionally distinct ER stress conditions have been identified. Firstly, apoptosis occurs when the functions of the ER are severely impaired [102, 103]. An increased rate of apoptosis has been observed in low-protein islets in vivo and in vitro [6, 9]. Secondly, translational attenuation possibly occurs to reduce the load of protein synthesis and to prevent further accumulation of unfolded proteins. Thirdly, activation of genes encoding ER chaperonins such as 78 M_r glucose-regulated protein (GRP78), also called immunoglobulin-binding protein (BiP), and glucose-regulated protein 94 (GRP94) could increase protein folding activity and prevent protein aggregation. The GRP78 is a member of the HSP70 family and synthesis is induced by a number of different stress conditions and by the accumulation of secretory precursors such as proinsulin in the ER [104]. It is weakly expressed in normal beta cells but is up-regulated in beta cells of mice with MODY, which develop hyperglycaemia with pancreatic beta-cell dysfunction. GRP78 was increased in the low-protein-diet islets. As GRP78 belongs to the HSP70 family, it could also have an anti-apoptotic role by inhibiting protein aggregation, decreasing formation of oxygen-free radicals and blocking caspases. It might also reduce necrosis by preventing ATP depletion [105]. Low ATP concentrations could be involved in cellular necrosis, because of ion pump failure, cellular swelling and plasma membrane rupture [106]. ATP synthase alpha chain, which is involved in ATP production, was down-regulated in the low-protein-diet islets.

Protein disulfide isomerase A3 is also called calreticulin. Overexpression increases the Ca (2+) content in the ER and protects against apoptosis mediated by nitric oxide [102]. Protein disulfide isomerase A3 s were identified in two spots on the gel, one up-regulated, the other down-regulated, which possibly reflects post-translatory modification of the protein and its activity. The same is true for the two up-regulated spots with T-complex protein-1 theta subunit (TCP-1), a type II chaperone up-regulated during recovery from chemical stress [107, 108].

Two proteins involved in cellular defence were modified and down-regulated in the low-protein-diet fetal islets, antioxidant protein 2 and calgranulin B. Antioxidant protein 2, also named 1-cys peroxiredoxin, can reduce hydrogen peroxide (H_2O_2). It could play a role in the protection against oxidative injury, as well as in the regulation of phospholipid turnover [109, 110]. If a reduced ability to protect against oxidative injury persists in adult life, beta cells in persons suffering intrauterine malnutrition could be more susceptible to free radicals.

Application of proteomics is a rapidly growing research area that encompasses both genetic and environmental factors. Our study shows that in Wistar rats, the intrauterine LP milieu may program islet gene expression in ways unfavourable for the future of the progeny. Indeed, after 1 week in culture fetal islet cells maintain alterations in proliferation, secretion and programmed cell death that can be explainable by the proteome analysis data in our study. The data do not allow distinction between primary and secondary changes either in time or in importance or between active and inactive isoforms of the proteins.

Whether the lasting consequences induced by the low-protein-diet in adult rats (e.g. lower beta-cell mass, lower plasma insulin concentrations and lower insulin secretion after a glucose challenge and increased susceptibility to cytokines) [9] can be directly related to changes in the fetal islet protein expression pattern described here is possible but not proven. Theoretically, this could be of importance for our understanding of the development of Type 1 and Type 2 diabetes mellitus.

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