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Placental Gene Expression Responses to Maternal Protein Restriction in the Mouse

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Abstract

OBJECTIVE—Maternal protein restriction has been shown to have deleterious effects on placental development, and has long-term consequences for the progeny. We tested the hypothesis that, by the use of microarray technology, we could identify specific genes and cellular pathways in the developing placenta that are responsive to maternal protein deprivation, and propose a potential mechanism for observed gene expression changes.

METHODS—We fed pregnant FVB/NJ mice from day post coitum 10.5 (DPC10.5) to DPC17.5, an isocaloric diet containing 50% less protein than normal chow. We used the Affymetrix Mouse 430A_2.0 array to measure gene expression changes in the placenta. We functionally annotated the regulated genes, and examined over-represented functional categories and performed pathway analysis. For selected genes, we confirmed the microarray results by use of qPCR.

RESULTS—We observed 244 probe sets, corresponding to 235 genes, regulated by protein restriction ($p < 0.001$), with ninety-one genes being up-regulated, and 153 down-regulated. Up-regulated genes included those involved in the p53 pathway, apoptosis, negative regulators of cell growth, negative regulators of cell metabolism and genes related to epigenetic control. Down-regulated genes included those involved in nucleotide metabolism.

CONCLUSIONS—Microarray analysis has allowed us to describe the genetic response to maternal protein deprivation in the mouse placenta. We observed that negative regulators of cell growth and metabolism in conjunction with genes involved in epigenesis were up-regulated, suggesting that protein deprivation may contribute to growth restriction and long-term epigenetic changes in stressed tissues and organs. The challenge will be to understand the cellular and molecular mechanisms of these gene expression responses.

Introduction

Successful placental development is crucial for optimal growth, maturation, and survival of the embryo/fetus. The placenta, a fetomaternal organ joining mother and offspring during pregnancy in mammals, serves as an endocrine organ in the “maternal-placental-fetal” complex, in addition to its role in the exchange of respiratory gases, exchange of nutrients, an immunologic barrier, and other functions. As has been recognized for many years, deviation in the normal gene expression pattern may lead to altered placental phenotype, as well as a modified phenotype of the conceptus. Previously, we have examined developmental gene expression patterns in the developing murine placenta, and reported numerous placental genes are up- or down-regulated to a significant degree, and that specific functional groups of genes are regulated at the different developmental ages [1] and with maternal hypoxia [2]. However,

a number of stressors during gestation can lead to altered placental and fetal growth and development. One of the important stressors is maternal malnutrition, which during pregnancy may have deleterious consequences for the progeny. Historical data point to these effects in human populations. For instance, during WWII, the people of both Holland and Russia were subjected to severe dietary restrictions due to interdiction of food supplies by the German army [3]. The children born under these conditions not only were small for gestational age, but they also developed significant health problems later in life [4,5]. Several major sequelae have been described including those of the cardiovascular system, type II diabetes, and mood and personality disorders [6].

Nutritional deprivation influences not only placental growth and morphology, but also alters the hormonal milieu of the developing fetus, and causes subsequent cardiovascular, hormonal and behavioral consequences in the adult [7,8]. These epidemiologic observations have led to speculation regarding the mechanism of changes in the placenta, and their effects on the developing fetus. The observations made in human subjects have been confirmed in several animal models. An important question, is the extent to which these observed effects result from an overall caloric restriction, as opposed to a qualitative component in the diet that triggers the responses. Evidence from several animal models points to protein deprivation as a major factor in these defects [9]. For example, in the rat the growth reducing effects of a low calorie diet can only be reversed by a dietary increase in protein levels; vitamin supplements, and caloric increases, while carbohydrates failed to reverse the observed effects [10]. Other studies have revealed that dietary amino acid balance is a key mediator of some of the cardiovascular and metabolic effects observed in response to protein deprivation [9]. However, no studies have examined the global changes in the placental gene expression with maternal protein restriction. We thus tested the hypothesis that, by the use of microarray technology, we could identify specific genes and cellular pathways in the developing placenta that are responsive to maternal protein deprivation, and propose a potential mechanism for phenotypic changes that have been observed.

Materials and Methods

Animals

Eight-week old FVB/NJ male and female mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed at the Animal Research Facility, Loma Linda University, Loma Linda, CA under conditions of 14 h light, 10 h darkness, ambient temperature of 20°C, and relative humidity of 30-60%. All experimental protocols were in compliance with the Animal Welfare Act, the National Institutes of Health *Guide for the Care and Use of Animals*, and were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Breeding and tissue collection

Mice were bred by overnight monogamous pairing of virgin females with a male, the male was removed in the morning, and that day was considered 0.5 day post coitum (0.5 dpc). Mice were weighed daily and pregnancy was confirmed by examining vaginal plugs on day 0.5 and weight gain by 10.5 dpc. At 17.5 dpc the pregnant females were euthanized. The uterus was removed rapidly and placed in a petri dish containing RNA Later solution (Ambion, Austin, TX). Entire placentae were isolated under a dissection microscope and maternal deciduas and endometrial tissues were removed. The isolated and cleaned placentae were snap frozen in liquid nitrogen, and stored at -80 °C for later analysis. RNA was isolated from the entire placentae using the TRIZOL reagent kit (Life Technologies, Rockville, MD), and was stored at -80 °C until further analysis. We confirmed the developmental stages of the embryos by visual inspection according to a modified Theiler staging system [11]. Details of the staging system are available online at <http://genex.hgu.mrc.ac.uk/Databases/Anatomy/MAstaging.html>.

Protein restriction

The mice were initially fed a normal mouse chow (20% protein content by weight, diet # TD91352). At 0.5 dpc the pregnant mice were divided into two groups, one group (n=3) were continued on normal mouse chow (control) and another group (n=3) were switched to a custom protein diet (10% protein by weight, diet # TD92208) (Teklad, Indianapolis, IA). The 50% protein deprivation was continued from 10.5 dpc to 17.5 dpc (total 7 days.). Studies in several species suggest that severe protein reduction leads to fetal programming of adulthood diseases in the offspring such as hypertension, schizophrenia, behavioral abnormalities etc [12-14]. Studies also indicate that maternal protein deprivation causes altered gene expression in different organs during different time points in the offspring lifespan and lead to these disorders. However, changes in the placental gene expression with this degree of protein deprivation are unknown, and were the focus of present study. The timing of the protein restriction was chosen in order to avoid interfering with fertilization and implantation of the embryo. We also sought to focus on the mature placenta, as in the mouse the allantoic fusion does not occur until 8 dpc and the placenta is not fully formed until 10.5 dpc. The diets were designed to ensure that mice would receive the same amount of calories and nutrients, but a reduced amount of protein. Maternal food intake and maternal weights were measured daily in order to assure isocaloric food intake.

Probe preparation, microarray hybridization, and data analysis

The RNA was processed for use on the Affymetrix Mouse 430A_2.0 array (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Briefly, 5 µg of total RNA was reverse transcribed to double stranded cDNA (Superscript II kit, Life Technologies). The double stranded cDNA was used in an in-vitro transcription reaction to generate biotinylated cRNA probes. The cRNA probes were purified, fragmented, and hybridized to the Affymetrix chip. Washes and staining were performed in an Affymetrix Gene Chip Fluidics station 400. The Affymetrix arrays were scanned using a Gene Array Scanner (Hewlett Packard, Austin, TX), and processed at the Microarray Facility, University of California Irvine, (Irvine, CA). The hybridizations were performed in triplicate for control and protein restricted conditions. All the placentas obtained from one mouse were pooled, and the total RNA isolated was considered as one RNA sample. Six such RNA samples, three each from protein restricted and control mice dams were used for microarray hybridization. Analyses were performed using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam (<http://linus.nci.nih.gov/BRBArrayTools.html>). We analyzed the data using the random variance method at a significance of $p < 0.001$ [15]. The genes were assigned to functional classes based on the GO database (<http://www.geneontology.org/GO.annotation.html>), and significantly over-represented GO categories in the gene sets were analyzed using the Gene Ontology Tree Machine (<http://genereg.ornl.gov/gotm/>). We also manually functionally annotated genes using Pubmed searches.

Real Time PCR

In an effort to validate the results of the microarray analysis, we chose several genes that were shown to be regulated by gestational protein restriction for analysis using real time PCR. RNA was isolated from mice different than the ones used for the microarray (n=5). Exon spanning primers were designed using the Universal Probe Library Assay Design Center (Roche, Indianapolis, IN). The primers were synthesized by Integrated DNA technologies (Coralville, CA). The primer sequences selected are shown in Table 1. Total RNA (1 µg per reaction) was reverse transcribed using random hexamers and the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). Relative expression was normalized to 18S RNA and fold changes were calculated using the $\Delta\Delta C_t$ method. Samples were analyzed on the Roche LightCycler 1.5 (Roche, Indianapolis, IN).

Results

In response to protein deprivation the placental weights remained unchanged while pup weights were significantly reduced ($p < 0.05$) as shown in Figure 1.

To evaluate the genetic response to protein deprivation we used the Affymetrix Mouse 430A_2.0 oligonucleotide array to compare gene expression levels between normal placentae at 17.5 dpc, and those from pregnancies in which the mothers were exposed to seven days of protein deprivation. Of 22,690 genes examined by on the microarray, using the random variance model [15], we observed 244 probe sets, corresponding to 235 genes, that were influenced by protein restriction ($p < 0.001$; some probe sets hybridize to different areas of the same gene. This is a design of the Affymetrix chip which serves as an internal control). As a consequence of maternal protein deprivation, 91 of these probe sets were up-regulated, while 153 were down-regulated. As noted in Table 2, among the gene ontology classes most over-represented in the up-regulated group, were regulators of apoptosis (Bcl2-like 2, p53, endophilin, Fas-activated serine/threonine kinase), negative regulators of cell growth (farnesyltransferase CAAX box beta, cadherin 5, CCAAT/enhancer binding protein (C/EBP) alpha, inositol polyphosphate-5-phosphatase D, p53), and negative regulators of cellular metabolism (nuclear receptor co-repressor 2, histone deacetylase 7A, SPEN homolog, transcriptional regulator). A number of genes involved in the p53 pathway were up-regulated. The genes *rai17* and *hipk2* were up-regulated, both of which are activators of p53. *Rai17* induces the expression of p53 and is a cofactor of p53-mediated gene regulation [16]. *Hipk2* is a kinase that phosphorylates Serine 46 on the p53 protein and activates its pro-apoptotic effects [17]. We also noted up-regulation of the gene *jmy*, a co-factor of p53. *Jmy* is up-regulated in response to DNA damage and binds to p53 in a protein complex that enhances its activity [18]. In addition we noted the up-regulation of two genes, *Cebpa* and *Inpp5d*, which are induced by p53. *Cebpa* is a leucine zipper transcription factor involved in the terminal differentiation of several cell types. It is up-regulated in response to UV radiation, and serves as a DNA damage induced G1 checkpoint in the cell [19]. *Inpp5d* is a phosphatase involved in inositol-mediated signaling, and has a potential anti-survival effect on the cell. It has been identified as a p53 transcriptional target [20].

Overall, the present study shows that the major pathways up-regulated with maternal protein deprivation are the p53 pathway, regulators of apoptosis, negative regulators of cell growth and metabolism and certain epigenetic regulators such as histone deacetylases, methionine adenosyl-transferase II alpha. In contrast, as noted in Table 3, among down-regulated genes, particularly striking were those genes related to nucleotide metabolism, and certain epigenetic regulators such as histone 2, *Mcm6* and telomeric repeat binding factor 1. The major placental gene pathways up- or down- regulated by maternal protein deprivation. We verified the expression of *Cebpa*, p53, *Rai17*, *Jmy*, *Hipk2* and *Inpp5d* by the use of real-time qPCR (Table 4). The expression of these genes were altered to similar extent as observed during our Microarray analysis.

Discussion

Microarray analysis is an invaluable tool to examine genetic mechanisms of cancer growth, development, responses to stress and, other processes. Numerous studies have been conducted utilizing this powerful tool of cDNA and oligo microarray, to elucidate the gene expression patterns in various physiological and pathological conditions. Previously, by the use of microarray analysis we have reported the changes in placental gene expression with fetal development [1] and acute maternal hypoxia [2]. In the present study we report the alterations in the placental gene expression with the maternal protein deprivation. Maternal protein restriction may play an important role in several disorders. Epidemiologic data in humans and

studies in laboratory animals provide useful lessons on the effects of caloric restriction/malnutrition on fetal development and disease prevalence in adulthood [4,5,12,21-26]. Those fetuses exposed to maternal caloric restriction in mid-gestation had a much greater incidence of bronchitis and other pulmonary disease [27] and renal disease as evidenced by microalbuminuria [28]. Females conceived during the famine also had a much higher prevalence of obesity as adults [4]. The cellular/molecular mechanisms of these in utero “programming” effects are unknown.

Studies in ruminants also have demonstrated that under-nutrition can have profound consequences for the fetus. In sheep, restricted maternal nutrition in early to mid-gestation was associated with an increase in placental weight, an increase in crown-rump length, and lower fetal to placental weight ratios [29]. Maternal under-nutrition also caused an alteration of cardiovascular homeostatic regulation by the renin-angiotensin system, and exposed the lambs to higher levels of glucocorticoids [30]. These hormonal effects also were associated with hypertension in the lambs [31]. Protein restriction in bovines also caused an increase in placental weight and an altered placental morphology [32].

Studies in rats have shown similar effects. Maternal protein restriction in rats triggers hypertension in the pups in adulthood. These effects appear to be mediated through a suppression of the renin-angiotensin system in the pups [33]. An alteration of placental glucocorticoid metabolism also was observed in placentae of rats fed a protein restricted diet. The activity of 11 β -hydroxysteroid dehydrogenase, an enzyme present in the placenta, which normally protects the pups from maternal glucocorticoid excess, was reduced in protein restricted rats [34]. Another hormonal alteration in nutritionally deprived rats was an increase in somatostatin expression in the periventricular nucleus of the pups. This led to much lower levels of growth hormone, and had deleterious effects on the growth of the pups post-partum [35]. Fetal undernourishment also led to neuronal sequelae. The facial motor nucleus in pups was under-developed, and led to a functional decrease in the ability of pups to suckle and chew.

A study somewhat similar to ours was conducted in the rat, revealing an increase in genes involved in apoptosis, and p53 [36]. A direct comparison of the results between the studies is difficult, however, because of intra-species differences and the different timing of the protein restriction. Nonetheless, it is of interest to note similar themes emerging. The earliest large-scale studies on caloric restriction were related to the slowdown of aging process in mice skeletal muscles [37]. In the present study, maternal protein restriction showed up-regulation of the genes responsible for the negative regulation of cell growth and metabolism in the placenta. It is of interest to observe that caloric or protein restriction in different tissues and at different ages effect similar groups of gene responsible for the decrease in cellular growth and metabolism. However, further studies are needed to examine the biologic mechanisms by which protein/caloric restriction produces up-regulation of this particular pathway.

In a previous study, we used the Affymetrix oligonucleotide array to define developmental changes in gene expression from 10.5 dpc to 17.5 dpc in the mouse placenta [1]. In addition, we have reported on significant changes in mouse placental gene expression in response to maternal hypoxia for 48 hours, from 15.5 dpc to 17.5 dpc [2]. In the present study of placental gene regulation in response to maternal dietary protein restriction, we demonstrate a profound down-regulation of cell growth and proliferation and an up-regulation of genes coding for apoptotic proteins (Tables 2 and 3). Of particular interest, p53 along with *rai17*, *Hipk2*, *jmy*, *Cebpa* and *Inpp5d* (proteins that either activate, or are cofactors of, or are induced by p53), an important regulator of cell growth and proliferation were up-regulated. This pathway serves as a G1 checkpoint, and arrests growth and/or induces apoptosis in response to cellular damage. Mutations in the p53 gene have been implicated in a number of cancers and other pathological processes [38]. *Hipk2*, an upstream regulator of p53, activates its transcriptional activity and

pro-apoptotic activities through phosphorylation at Ser 46 [17]. Cebpa, a transcription factor induced by p53, mediates some of the downstream effects of p53 activation [19]. Several studies on the effects of nutritional deprivation have demonstrated that the p53 pathway is a crucial mediator of the observed biological effects. Mice deficient in p53 (p53 ^{-/-}) are more susceptible to cancer, but caloric restriction partially reversed that effect [39].

The present study has demonstrated a significant up-regulation of genes responsible for apoptosis regulation such as Bcl2-like 2, p53, endophilin, Fas-activated serine/threonine kinase. Apoptosis and its associated regulatory mechanisms are physiological events crucial to the maintenance of homeostasis in the placenta and other organs. Imbalance of these processes may cause various pathological conditions, may compromise placental function and, consequently, pregnancy success. Increased apoptosis occurs in the placentas of pregnant women with several developmental abnormalities, while increased Bcl-2 expression is generally associated with pregnancy-associated tumors and decreased expression is associated with placentas of the diabetic women [40]. Another important finding of the study was upregulation of Fas-activated serine/threonine phosphoprotein (FAST). FAST is a survival protein, bound to the outer mitochondrial membrane and mediates alternative and constitutive splicing, which may affect the expression of several other genes [41].

A potentially important finding of the present study, is that protein deprivation altered the expression of several genes involved in DNA methylation and histone acetylation, which are involved in epigenetic regulation of gene expression. The expression levels of histone deacetylase 7A and methionine adenosyltransferase II alpha, were significantly elevated. Histone acetylation triggers changes in chromatin structure, and regulates transcriptional availability of genes. In turn, histone deacetylation increases histone affinity for DNA, thereby repressing transcription. Methionine adenosyltransferase II alpha synthesizes AdoMet, the direct precursor used for DNA methylation by methyltransferases. Histone 2 (h3c2) is down-regulated, along with Mcm6 and telomeric repeat binding factor 1. These proteins contribute to DNA replication, stability, and structure [42,43]. Recent studies in the human, have demonstrated in small for gestational age pregnancies an altered DNA methylation pattern in imprinted regions of the genome, and that imprinted genes are expressed in an unbalanced manner in pregnancies affected by intrauterine growth retardation [44,45].

In several animal models, in addition to potential deleterious effects, a positive aspect of nutritional deprivation in the adult is that of prolonged lifespan and reduced cancer rates. A proposed mechanism for these benefits is that nutritional restriction in the absence of malnutrition inhibits cellular proliferation and induces apoptosis. This effect has been shown in mice lacking p53, in which ^{-/-} and ^{+/-} mutants have lowered spontaneous cancer rates when fed a complete, but calorically reduced diet [39]. In the adult and aging animal, nutritional restriction has been shown to have beneficial effects that increased life span [46]. In contrast, a different picture has emerged in the fetus. As discussed above, caloric and protein deprivation have been shown to trigger fetal programming of adult disease, and lead to an increased prevalence of metabolic disorders in adulthood. In the developing fetus, numerous animal studies have shown the negative long-term effects of caloric and protein deprivation on the cardiovascular, renal, nervous system and metabolism (for review see [47]). Both fetal and placental growths are essential for the long-term well-being of the individual. Thus, one would anticipate that profound inhibition of cellular growth at key time points during development would have grave long-term consequences for the embryo/fetus. This suggests that the timing of the treatment is a key determinant in the effect on the organism.

Perspective/Conclusions

The present data support the hypothesis that maternal protein restriction triggers an up-regulation of apoptosis-related genes, an increase in the p53 pathway, a change in epigenetic modulators, and an overall down-regulation of cellular proliferation and growth associated genes. The upregulation of inhibitory transcription factors, and other key negative cellular regulators altered by protein deprivation, offers a picture of profound and global down-regulation of the entire cellular proliferative machinery. These results suggest numerous avenues for future research, and raise a number of fundamental questions regarding energy/protein balance and cellular growth. A critical challenge will be to understand the cellular and molecular mechanisms of these epigenetic responses.

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Figure 1.
Pup and placental weights at 17.5 dpc after 50% protein restriction (* $p < 0.05$).

Table 1

Primers used for qPCR to verify expression of selected genes

Gene Name Accession Number	Forward Primer (Position)	Reverse Primer (Position)	Amplicon Length
Rai 17 NM_183208	gagacaagttcaccccaag (753-772)	ggccaagttcttcacacca (794-812)	60
p53 NM_001127233	gcccattgctacagaggagtc (1206-1225)	agactggcccttcttggtct (1257 - 1276)	72
Cebpa NM_007678	ccttcaacgacgagttcctg (331 - 350)	tggccttctcctgctgctc (373 - 390)	60
Jmy NM_021310	aagggtatgaagaggtgctt (1341 - 1361)	ctttctatagcttctgtgtcca (1393 - 1417)	77
Hipk2 NM_010433	cagcagtgacaccgatgaag (2806 - 2825)	tctttgcttgagactgtgc (2853 - 2872)	67
18S NR_003278.1	ctcaacacgggaaacctcac (1247-1266)	cgctccaccaactaagaacg (1337-1356)	110

Table 2

Genes Up-regulated by protein deprivation

Fold Change	Probe Set	Gene Description
1.86	1451895_a_at	24-dehydrocholesterol reductase
1.776	1427385_s_at	actinin, alpha 1
1.435	1433477_at	active BCR-related gene
1.648	1419140_at	activin receptor IIB
1.378	1423973_a_at	ADP-ribosylation factor 3
1.728	1438501_at	ribosomal protein S17
1.9	1423556_at	aldo-keto reductase family 1, member B7
1.745	1424956_at	AT hook, DNA binding motif, containing 1
1.463	1423526_at	AT rich interactive domain 3B (Bright like)
1.532	1425227_a_at	ATPase, H ⁺ transporting, lysosomal V0 subunit a isoform 1
1.411	1427565_a_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 5
1.537	1430453_a_at	Bcl2-like 2
1.723	1423816_at	CAAX box 1 homolog B (human)
2.539	1422047_at	cadherin 5
1.628	1451896_a_at	calcium homeostasis endoplasmic reticulum protein
1.504	1417175_at	casein kinase 1, epsilon
1.603	1422450_at	catenin (cadherin associated protein), delta 1
1.538	1418982_at	CCAAT/enhancer binding protein (CEB/P), alpha
2.01	1451359_at	cDNA sequence BC005662
1.421	1424726_at	cDNA sequence BC014685
1.635	1460034_at	cDNA sequence BC042901
1.467	1419833_s_at	centaurin, delta 3
1.433	1448274_at	complement component 1, q subcomponent binding protein
1.52	1425204_s_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 19a
1.743	1421143_at	diaphanous homolog 1 (Drosophila)
1.637	1450475_at	distal-less homeobox 3
1.376	1419502_at	DNA segment, Chr 11, Lothar Hennighausen 1, expressed
1.416	1421032_a_at	DnaJ (Hsp40) homolog, subfamily B, member 12
1.893	1452100_at	Dullard homolog (Xenopus laevis)
1.497	1418648_at	EGL nine homolog 3 (C. elegans)
1.461	1425788_a_at	enoyl Coenzyme A hydratase domain containing 2
1.64	1427039_at	epsin 1
1.454	1452273_at	expressed sequence AA409316
1.64	1424428_at	expressed sequence AI225782
1.631	1420602_a_at	extraembryonic, spermatogenesis, homeobox 1
1.937	1434309_at	farnesyltransferase, CAAX box, beta
1.439	1460635_at	Fas-activated serine/threonine kinase
1.553	1449849_a_at	F-box and leucine-rich repeat protein 6
1.395	1423442_a_at	F-box and WD-40 domain protein 2
1.496	1418396_at	G-protein signalling modulator 3 (AGS3-like, C. elegans)
1.457	1424101_at	heterogeneous nuclear ribonucleoprotein L

Fold Change	Probe Set	Gene Description
1.728	1417637_a_at	high mobility group 20 B
1.618	1420813_at	histone deacetylase 7A
1.825	1422799_at	HLA-B associated transcript 2
2.829	1429566_a_at	homeodomain interacting protein kinase 2
1.576	1424195_a_at	inositol polyphosphate-5-phosphatase D
2.023	1418265_s_at	interferon regulatory factor 2
1.378	1448759_at	interleukin 2 receptor, beta chain
1.879	1448668_a_at	interleukin-1 receptor-associated kinase 1
1.572	1452327_at	IQ motif and Sec7 domain 1
1.515	1426873_s_at	junction plakoglobin
1.497	1420639_at	junction-mediating and regulatory protein
1.548	1428881_at	kinesin 2
1.422	1451066_at	leukocyte receptor cluster (LRC) member 4
1.551	1426648_at	MAP kinase-activated protein kinase 2
1.625	1456618_at	MAP/microtubule affinity-regulating kinase 4
1.493	1437226_x_at	MARCKS-like 1
1.519	1456702_x_at	methionine adenosyltransferase II, alpha
1.392	1417857_at	methylmalonic aciduria (cobalamin deficiency) type A
1.56	1417472_at	myosin, heavy polypeptide 9, non-muscle
1.438	1452254_at	myotubularin related protein 9
1.446	1428367_at	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1
1.963	1448893_at	nuclear receptor co-repressor 2
2.067	1424952_at	OCIA domain containing 1
2.138	1423967_at	paralemmi
2.745	1427550_at	paternally expressed 10
1.918	1449851_at	period homolog 1 (Drosophila)
1.58	1438938_x_at	prohibitin 2
1.662	1418078_at	proteasome (prosome, macropain) 28 subunit, 3
1.658	1418341_at	RAB4A, member RAS oncogene family
1.707	1450903_at	RAD23b homolog (S. cerevisiae)
2.93	1418535_at	ral guanine nucleotide dissociation stimulator,-like 1
1.585	1425266_a_at	RAP1, GTP-GDP dissociation stimulator 1
5.137	1455984_at	retinoic acid induced 17
1.888	1438507_x_at	ribosomal protein L14
1.613	1416033_at	RIKEN cDNA 1110006I15 gene
1.909	1431213_a_at	RIKEN cDNA 1300007C21 gene
2.844	1460609_at	RIKEN cDNA 2810013E07 gene
1.922	1431753_x_at	RIKEN cDNA 2900073H19 gene
1.677	1427979_at	RIKEN cDNA 4732418C07 gene
1.786	1425000_s_at	RIKEN cDNA 5430407P10 gene
1.863	1437180_at	RIKEN cDNA 6530403A03 gene
1.411	1448251_at	RIKEN cDNA 9030425E11 gene

Fold Change	Probe Set	Gene Description
1.775	1452351_at	RIKEN cDNA C030027K23 gene
1.378	1436906_at	ring finger protein 166
2.115	1450918_s_at	Rous sarcoma oncogene
1.717	1434438_at	SAM domain and HD domain, 1
1.422	1416294_at	secretory carrier membrane protein 3
1.902	1422167_at	semaphoring 5A
1.555	1418011_a_at	SH3-domain GRB2-like B1 (endophilin)
1.891	1449005_at	solute carrier family 16 (monocarboxylic acid transporters), member 3
1.954	1452139_at	solute carrier family 35, member C1
1.872	1418326_at	solute carrier family 7 (cationic amino acid transporter, y+system), member 5
1.713	1419329_at	sorbin and SH3 domain containing 3
1.768	1420397_a_at	SPEN homolog, transcriptional regulator (Drosophila)
1.808	1425787_a_at	synaptotagmin-like 3
1.871	1423616_at	TAR (HIV) RNA binding protein 2
1.603	1420593_a_at	TEA domain family member 3
1.612	1451586_at	testis enhanced gene transcript
1.57	1452768_at	testis expressed gene 261
1.475	1419954_s_at	testis expressed gene 27
1.828	1421148_a_at	Tial1 cytotoxic granule-associated RNA binding protein-like 1
2.282	1460743_at	tigger transposable element derived 5
1.371	1426691_at	tight junction associated protein 1
1.471	1426610_a_at	transcription termination factor 1
1.649	1426538_a_at	transformation related protein 53
1.759	1448412_a_at	TSC22 domain family 4
1.991	1451771_at	two pore channel 1
1.658	1448370_at	Unc-51 like kinase 1 (C. elegans)
2.773	1423726_at	vesicle amine transport protein 1 homolog (T californica)
1.878	1420834_at	vesicle-associated membrane protein 2
1.722	1448121_at	WW domain binding protein 2
1.631	1423750_a_at	Zinc finger protein 162, mRNA (cDNA clone MGC:7095 IMAGE:3157495)
2.533	1418670_s_at	Unknown

Table 3

Genes down-regulated by protein deprivation

Fold Change	Probe Set	Gene Description
0.463	1422716_a_at	acid phosphatase 1, soluble
0.697	1438170_x_at	adhesion regulating molecule 1
0.637	1423781_at	amyloid beta precursor protein binding protein 1
0.655	1437688_x_at	ATPase, H ⁺ transporting, lysosomal accessory protein 2
0.724	1451223_a_at	basic transcription factor 3-like 4
0.728	1450732_a_at	bicaudal D homolog 2 (Drosophila)
0.636	AFFX-BioB-M_at	Biotin synthase /// biotin synthesis, sulfur insertion?
0.585	1436885_a_at	calcium homeostasis endoplasmic reticulum protein
0.663	1437670_x_at	CD151 antigen
0.508	1451232_at	CD151 antigen
0.584	1425646_at	cDNA sequence BC016495
0.65	1419403_at	cDNA sequence BC017612
0.724	1423682_a_at	cell division cycle associated 4
0.642	1436390_a_at	chloride channel CLIC-like 1
0.577	1433718_a_at	chromobox homolog 1 (Drosophila HP1 beta)
0.498	1436838_x_at	coactosin-like 1 (Dictyostelium)
0.51	1454781_x_at	COMM domain containing 9
0.398	1437982_x_at	COX15 homolog, cytochrome c oxidase assembly protein (yeast)
0.717	1434705_at	C-terminal binding protein 2, mRNA (cDNA clone MGC:27651 IMAGE:4511826)
0.655	1454149_a_at	cyclin L2
0.605	1438371_x_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
0.673	AFFX-r2-Ec-bioD-3_at	dethiobiotin synthetase
0.631	1419915_at	DNA segment, Chr 10, ERATO Doi 438, expressed, mRNA (cDNA clone MGC:7199 IMAGE:3482163)
0.637	1449339_at	DNA segment, Chr 10, ERATO Doi 641, expressed
0.638	1429411_a_at	enhancer of yellow 2 homolog (Drosophila)
0.566	1416236_a_at	epithelial V-like antigen 1
0.652	1424013_at	eukaryotic translation termination factor 1
0.657	1439411_a_at	exportin 7
0.596	1455912_x_at	Expressed sequence AW538196 (AW538196), mRNA
0.679	1434108_at	F-box protein 11
0.656	1452247_at	fragile X mental retardation gene 1, autosomal homolog
0.628	1439150_x_at	GH regulated TBC protein 1, mRNA (cDNA clone MGC:27905 IMAGE:3500563)
0.641	1419072_at	glutathione S-transferase, mu 7
0.697	1424030_at	grainyhead-like 1 (Drosophila)
0.483	1416855_at	growth arrest specific 1
0.531	1418106_at	hairy/enhancer-of-split related with YRPW motif 2
0.522	1434047_x_at	heterogeneous nuclear ribonucleoprotein A2/B1
0.36	1437099_x_at	Heterogeneous nuclear ribonucleoprotein F, mRNA
0.611	1422155_at	(cDNA clone MGC:36543 IMAGE:4950131) histone 2, H3c2

Fold Change	Probe Set	Gene Description
0.618	1455777_x_at	hydroxysteroid (17-beta) dehydrogenase 4
0.438	1455930_at	hypothetical LOC433566 /// hypothetical LOC434498
0.486	1423033_at	intergral membrane protein 1
0.555	1449099_at	LPS-responsive beige-like anchor
0.712	1416343_a_at	lysosomal membrane glycoprotein 2
0.44	1455978_a_at	matrilin 2
0.686	1434378_a_at	Max dimerization protein 4, mRNA (cDNA clone MGC:19425 IMAGE:3490469)
0.651	1422498_at	melanoma antigen, family H, 1
0.597	1438852_x_at	minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)
0.565	1455787_x_at	multiple inositol polyphosphate histidine phosphatase 1
0.596	1456381_x_at	myeloid cell leukemia sequence 1
0.553	1456028_x_at	Myristoylated alanine rich protein kinase C substrate (Marcks), mRNA
0.341	1438955_x_at	peptidylprolyl isomerase F (cyclophilin F)
0.637	1455860_at	phosphatidylinositol glycan, class H
0.356	1420132_s_at	Pituitary tumor-transforming 1 interacting protein, mRNA (cDNA clone MGC:38220 IMAGE:5323397)
0.658	1425721_at	pleckstrin homology domain interacting protein
0.658	1451740_at	polyadenylate binding protein-interacting protein 1
0.51	1456270_s_at	preferentially expressed antigen in melanoma like 6
0.629	1437845_x_at	protein O-fucosyltransferase 2
0.665	1451225_at	protein tyrosine phosphatase, non-receptor type 11
0.678	1455105_at	protein tyrosine phosphatase, non-receptor type 12
0.728	1419069_at	RAB guanine nucleotide exchange factor (GEF) 1
0.471	1419946_s_at	RAB2, member RAS oncogene family (Rab2), mRNA
0.506	1455809_x_at	resistance to inhibitors of cholinesterase 8 homolog (C. elegans)
0.483	1426604_at	ribonuclease L (2', 5'-oligoadenylate synthetase-dependent)
0.489	1418337_at	ribose 5-phosphate isomerase A
0.665	1437246_x_at	ribosomal protein S6 /// similar to 40S ribosomal protein S6
0.644	1417222_a_at	RIKEN cDNA 2310075C12 gene
0.636	1452167_at	RIKEN cDNA 2810407C02 gene
0.657	1426986_at	RIKEN cDNA 2810485I05 gene
0.645	1418173_at	RIKEN cDNA 4631426H08 gene
0.315	1418997_at	RIKEN cDNA 4930469P12 gene
0.488	1456582_x_at	RIKEN cDNA 5230400G24 gene
0.711	1435240_at	RIKEN cDNA 5830435C13 gene
0.647	1440831_at	RIKEN cDNA 6230421P05 gene
0.474	1436213_a_at	RIKEN cDNA C430010P07 gene
0.656	1448434_at	ring finger protein 103
0.711	1423740_a_at	RNA binding motif protein 10
0.687	1419977_s_at	RNA binding motif protein 35b
0.48	1420982_at	RNA-binding region (RNP1, RRM) containing 2
0.695	1437461_s_at	RNA-binding region (RNP1, RRM) containing 3

Fold Change	Probe Set	Gene Description
0.63	1437995_x_at	septin 7
0.605	1418422_at	serine (or cysteine) peptidase inhibitor, clade B, member 9g
0.489	1419913_at	Serine/threonine kinase receptor associated protein (Strap), mRNA
0.659	1416041_at	serum/glucocorticoid regulated kinase
0.676	1416862_at	signal transducing adaptor molecule (SH3 domain and ITAM motif) 1
0.666	1439433_a_at	solute carrier family 35 (UDP-galactose transporter), member 2
0.66	1452281_at	Son of sevenless homolog 2 (Drosophila)
0.67	1454794_at	spastin
0.585	1417300_at	sphingomyelin phosphodiesterase, acid-like 3B
0.727	1436809_a_at	spindlin
0.564	1455899_x_at	suppressor of cytokine signaling 3
0.485	1420175_at	Tax1 (human T-cell leukemia virus type I) binding protein 1, mRNA (cDNA clone MGC:11692 IMAGE:3962810)
0.612	1431332_a_at	telomeric repeat binding factor 1
0.626	1415908_at	testis-specific protein, Y-encoded-like 1
0.536	1437454_a_at	thioredoxin domain containing 14 /// similar to thioredoxin-related transmembrane protein 2
0.366	1420042_at	THO complex 1
0.462	1422781_at	toll-like receptor 3
0.625	1437729_at	Transcribed locus, strongly similar to XP_484309.1 PREDICTED: similar to ribosomal protein L27A [Mus musculus]
0.59	1436392_s_at	transcription factor AP-2, gamma
0.372	1449671_at	Transmembrane 7 superfamily member 1 (Tm7sf1), mRNA
0.454	1419918_at	transmembrane emp24 protein transport domain containing 7
0.535	1439444_x_at	transmembrane emp24-like trafficking protein 10 (yeast)
0.513	1435064_a_at	transmembrane protein 27
0.693	1428586_at	transmembrane protein 41B
0.6	1424275_s_at	tripartite motif-containing 41
0.562	1425562_s_at	tRNA nucleotidyl transferase, CCA-adding, 1
0.709	1438855_x_at	tumor necrosis factor, alpha-induced protein 2
0.518	1439005_x_at	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz), mRNA
0.682	1437714_x_at	Ubiquitin specific peptidase 14, mRNA (cDNA clone MGC:7106 IMAGE:3157723)
0.522	1452011_a_at	UDP-glucuronate decarboxylase 1
0.615	1421050_at	vacuolar protein sorting 25 (yeast)
0.708	1457285_at	zinc finger protein 187
0.613	1419181_at	zinc finger protein 326
0.513	1427590_at	zinc finger protein 39
0.426	1449691_at	Zinc finger protein 644, mRNA (cDNA clone IMAGE:4037170)
0.511	1451518_at	zinc finger protein 709
0.586	AFFX-r2-Bs-phe-3_at	Unknown
0.585	AFFX-ThrX-M_at	Unknown
0.575	AFFX-DapX-M_at	Unknown

Fold Change	Probe Set	Gene Description
0.571	1419997_at	Unknown
0.512	1424746_at	Unknown
0.471	1447977_x_at	Unknown

Table 4

qPCR validation of selected genes

Gene Name	Fold Change by Microarray	Avg ddCT	Mean Fold Change by qPCR (SEM)
Cebpa	1.6	-0.93	1.9 (0.21)
p53	1.5	-1.14	2.1 (0.33)
Rai17	5.1	-1.135	2.1 (0.41)
Jmy	1.5	-1.15	2.2 (0.27)
Hipk2	2.8	-1.13	2.1 (0.22)
Inpp5d	1.6	-0.71	1.6 (0.18)