Placenta 30 (2009) 411–417

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Placental Gene Expression Responses to Maternal Protein Restriction in the Mouse

C.P. Gheorghe [a], R. Goyal [a], J.D. Holweger [a], L.D. Longo [a,b,*]

[a] Department of Physiology, Loma Linda University, School of Medicine, Loma Linda, CA 92350, United States
[b] Department of Obstetrics and Gynecology, Loma Linda University, School of Medicine, Loma Linda, CA 92350, United States

ARTICLE INFO

Article history:
Accepted 5 March 2009

Keywords:
Placenta
Gene regulation
Microarray
Protein restriction
p53

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.

1. Introduction

Successful placental development is crucial for optimal growth, maturation, and survival of the embryo/fetus. The placenta, a feto-maternal 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].

* Corresponding author. Department of Physiology, Loma Linda University, School of Medicine, Loma Linda, CA 92350, United States. Tel.: +1 909 558 4325; fax: +1 909 558 4029.
E-mail address: llongo@llu.edu (L.D. Longo).

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doi:10.1016/j.placenta.2009.03.002

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.

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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.

2.4. 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 biotynilated 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/BRB-ArrayTools.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.

2.5. 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 one 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 ΔΔCt method. Samples were analyzed on the Roche LightCycler 1.5 (Roche, Indianapolis, IN).

3. Results

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