FOOD-CT-2005-007036

EARNEST

EARly Nutrition programming- long term follow up of Efficacy and Safety Trials and integrated epidemiological, genetic, animal, consumer and economic research

Instrument: Integrated Project

Thematic Priority 5.4.3.1: Food Quality and Safety

Final public report on activity 3.5.1:

Animal and cell culture studies and genomics

Period covered from 15.04.2005 to 14.10.2010

Start date of project: 15.04.2005 Duration: 5,5 Years

Organisation Name of Lead Contractor for this report: RRI

ACTIVITY OBJECTIVES AND MAIN TASKS

This work package aimed to establish links with the European Nutriogenomics Network (NuGO) a European Union funded Network of Excellence. Collaboration with this NuGO allowed EARNEST partners access to up to the minute technologies and essential statistical and bioinformatics expertise.

The initial objective of this activity was to build a placental gene array, to test and to use them to obtain genomic information concerning placental cells grown in culture as the pilot study and then placental tissue from rodent models being used by EARNEST to study fetal programming.

The placental cells that we use as a model for the placenta are the human choriocarcinoma cell line, BeWo. The conditions for using this cell line in dual-chamber culture systems which allow the study not only nutrient uptake but also transport across the cell layer needed to be established. In collaboration with NuGO colleagues we carried out detailed evaluation of the structural and functional properties of BeWo (b30 subclone) cell layers grown on permeable supports with respect to their use for placental iron transport studies. Secondly we wanted to determine whether it is feasible to co-culture a human liver hepatocellular carcinoma HepG2 cells and BeWo cells, to mimic the in vivo situation in pregnancy were these organs are known to communicate and respond to hormone and cytokine signals released by the other.

The original approach of developing a human placental array was overtaken rapidly by new developments resulting from our collaboration with the NuGO. Through commercial partners within NuGO this EARNEST activity was allowed access to whole gene arrays at a discount which made the developing and using our on placental specific array not cost effective.

The deliverables original established for this activity were modified to include the use of these arrays. However the main aims remained the same obtaining data on the differential gene expression in micronutrient deficiencies and the identification of genes and gene pathways responsible for the induction of hypertension and other fetal programming effects.

RESULTS

Iron transfer across BeWo cell layers (Deliverables 47-49)

BeWo cells are a placental cell line that has been widely used as an in vitro model for the placenta. The b30 subclone of these cells can be grown on permeable membranes in bicameral chambers to form confluent cell layers, enabling rates of both nutrient uptake into the cells from the apical surface and efflux from the basolateral membrane to be determined.
Importantly, this study has shown that BeWo cells may not form confluent monolayers. We showed that the the cells form only a transient monolayer, before forming cell multilayers. The integrity of the cell layer is critical if the BeWo cells grown on membrane supports are to be used for nutrient transport studies. Therefore, we decided that the most robust option was to work with the cell multilayers rather than risk the presence of gaps in a cell monolayer.
Under these conditions, we were able to demonstrate the presence of tight junctions between the cells, confirming the validity of the TEER measurements.

The fact that BeWo cells may form more complex structures than simple monolayers means that data interpretation is more difficult than originally anticipated. Mathematical modeling of the forward transport data from experiments using the revised protocol provided an overview of the apparent transport mechanism of Tf-bound iron in the BeWo cell system. This matched perfectly with the known aspects of Tf receptor-mediated uptake of iron in

which holo-Tf binds to the Tf receptor and is internalized. Iron is released from the receptor complex within endosomes as a result of acidification of the endosomal environment. The free iron is removed from the endosomes for transport into and across the cell, while the apo-Tf is recycled together with the Tf-receptor predominantly back to the cell surface, where it is released again. The best-fit models suggested that all the iron passing through the cell layer was doing so via this transcellular process. The model derived from the reverse Fe-Tf transport data suggested that iron also can be transported from the basolateral side, through the cells, to the apical compartment in much the same way that it is transported in the opposite direction.

Placental and liver co-cultures, using the dual chamber culture system, were established in order to test the hypothesis that having cells in the basal compartment would reduce the level of iron in that medium. This would result in a larger gradient between the maternal and fetal sides of the placenta. We would then expect, depending on how the transporter systems were regulated, that there would been increase in the transfer rate. The method involved seeding the base of a 3.5 cm culture dish with HepG2 cells – acting as the fetal liver cells, then adding a suspended filter layered with BeWo cells. In these experiments, transfer of iron only was measured. Differences between passive and active transport was determined using temperature sensitivity.

As would be expected, the amount of iron transferred increased at 37°C when compared to 4°C. The amount was also remarkably high. Within the 6 h incubation period, nearly 50 % of the iron was moved from apical to basolateral sides. The amount of iron transferred through the BeWo cells in the presence of HepG2 cells on the base of the chamber was markedly reduced. This was not what we expected. The difference could not be accounted for by accumulation of ^{59}Fe in the HepG2 cells but suggest that the presence of HepG2 cells in the basal layer inhibits the uptake and transfer of ^{59}Fe by the BeWo cells. There is clearly an active transport component, but the fact that the hepatocytes can decrease the transfer rate, suggests that the HepG2 cells are secreting an inhibitory factor. To verify if this was the case, we conducted a time course experiment (Fig 1). The data do suggest that HepG2 cells inhibit the transfer of iron across the BeWo cell layer. How and why HepG2

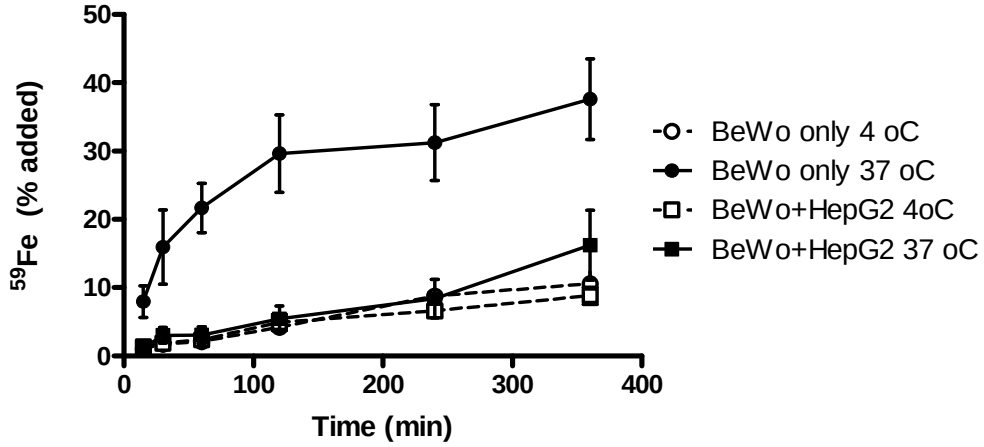

Fig. 1. Transfer of ^{59}Fe across the BeWo cell layer in the presence and absence of HepG2 cells

cells are able to regulate the transport of iron across BeWo cells is not clear. We believe the liver cells signal their normal iron status, to the placental cells in order to maintain adequate iron supplies; in this case they are indicating a reduced Fe requirement. It is possible that the HepG2 cells produce hepcidin, which in turn may inhibit trans-cellular transfer.

Differential gene expression in placenta; a result of maternal micronutrient deficiencies (Deliverables 50, 81-83)

As a result of the establishment of a formal link between EARNEST and the European Network of Excellence, NuGO, not only were we able to use Affymetrix rat specific whole genome arrays but we were also allowed access to the bioinformatics resources of NuGO. NuGO had organised for network members to have access to not only a provider who would run the gene arrays, bit access to specialist software for analysing the results including quality assurance, bioinformatics and statistical analysis, this became known as the NuGO 'array pipeline'. An initial study was carried out comparing the micronutrient induced changes in gene expression in placenta allowing us to establish the necessary protocols and procedures for EARNEST members to carry out gene array analysis using this 'array pipeline.

Isolated RNA, from control, iron deficient and copper deficient placenta, was sent for array analysis. Hybridisations were carried out using Affymetrix whole rat genome arrays (230 2.0 arrays). The resulting data was uploaded to the 'MadMax' server which carries out all quality assurance, both for samples and arrays, and normalisations. The data is downloaded to Excel and "cef" files, which show the changes in expression across the entire genome. The 'array pipeline' worked well and the protocols and procedures established under deliverable 82, were not only used by this workpackage in further activities but also by work package 3.3.

Studying the initial array data led to some interesting observations (Fig 2). Of the 31000 genes represented on the array only 4% were expressed above background levels in the placenta. The data was the filtered according to p value (cut off $p<0.05$) and fold change (cut off >1.2, <-1.2) to determine which genes show differential expression. As expected, we did not observe huge fold change differences and therefore we are just looking for the slight physiological changes. For this analysis we also used the p-adjusted values to reduce the number of false positives, so we could be confident that the genes that appear on this list are truly affected. For each diet about 500 genes showed significant differential expression.

Fig 2. The effect of iron (red pie chart) and copper (green pie chart on gene expression in the rat placenta. The separated, expanded, section on the left shows those genes that have expression above background in the placenta.

This initial data set could be analysed as two separate studies. Firstly by comparing each deficiency to its control, this allows us to determine how each micronutrient alters gene expression. Secondly we can compare the genes with altered expression caused by each deficiency, this will allow us to identify genes that are sensitive to more than one nutrient. The resulting gene expression data for each study can then be analysed by two different methods; either by complying lists in a programme such as Microsoft Excel or by using pathway analysis programmes such as Metacore^{TM}.

By using both the fold change and p adjusted values greatly reduced the number of common genes considered targets, a list of ten genes of interest is given in table 1. Transferrin Receptor acts as a positive control, we know this should be up-regulated under both conditions. There are seven that are up-regulated and three that are down regulated. The

biological function of all these genes are known, they include, as expected genes involved in ion and metal transporters, but also genes involved in transcription, DNA repair and lipid metabolism.

Gene Id	Description	Direction of expression
Tfrc	Blood plasma protein for iron delivery	Up
DMT1	Transporter involved in iron metabolism	Up
Cacna1e	Calcium voltage dependent channel	Up
Prdm2	Zinc finger protein, transcription factor	Up
Bmp1	Calcium binding	Up
Cln3	G1 cyclin involved in cell cycle progression	Up
hHR23a	DNA repair and proteolytic pathways	Up
H2AFO	Transcriptional regulation	Up
EMSY	Role in chromatin remodelling	Down
Gosr1	SNAP receptor intra-golgi transport	Down
Snrpd1	Transcription factor regulation	Down

Table 1. Genes affected in common. Placental genes which showed significant gene expression changes in response to both iron and copper deficient diets.

Through NuGO we have access to several different pathway analysis tools; we have used Metacore^{TM} to help to identify the most significantly affected metabolic pathways. By using this software we are able to obtain lists of biochemical pathways which contain genes that have significant gene expression changes. The pathways are ranked taking into account the number of genes changed in the pathway, the size of change and the significance value attributed to that change. When the list of pathways altered in both iron and copper deficiency was studied it became clear that the effects of micronutrient deficiency were far reaching (Fig 3). The pathways identified were involved in amino acid metabolism, lipid metabolism through to cytoskeletal organisation.

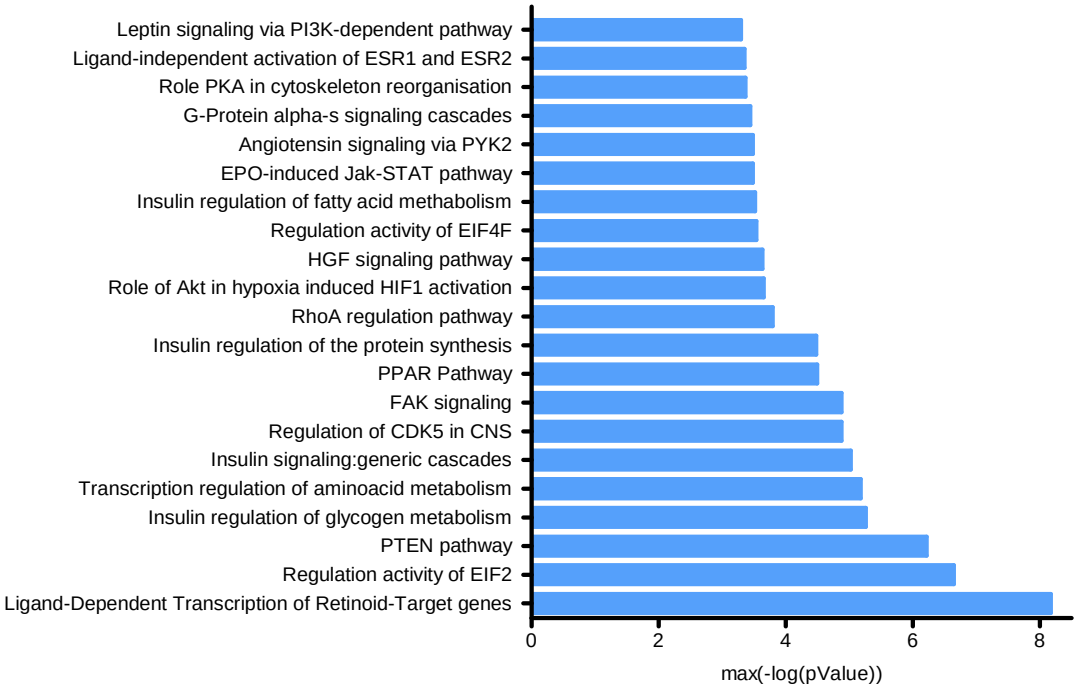

Fig 3. Metabolic pathways sensitive to both iron and copper deficiency.

The initial array analysis carried out on the iron deficient and copper deficient placenta clearly indicated that analysis of target tissue via whole genome array analysis would generate a vast number of target genes and pathways. Therefore in order to determine which genes or pathways were responsible for the generation of hypertension, it was concluded that we need to add other models to our analysis. Therefore placental samples were obtained from consortium members working on both low protein and high fat models of fetal programming. The data generated by this study will be presented in workpackage 3.5.2.

CONCULSIONS

Iron transfer across BeWo cell layers

The BeWo placental cell line is widely used as an in vitro model for the placenta; therefore the technical data obtained in this workpackage are important to a number of groups in the scisntific community. Our results have shown that there is only a narrow window when these cells form monolayers and a decision has to be made at the start of each study to either limit studies to this small window or to work with the cells after they formed multilayers. Further studies of the processes which occur as the cells overlay each other are needed. These were outwith the scope of the current project, but we will apply for support to continue it from other funders. The placental and liver cell line co-cultures, using the dual chamber culture system, were established in order to determine whether placental liver interactions could be studied in vitro. Such studies were expected to be relatively easy to manipulate when compared to in vivo studies in animals. However the method turned out to be more complex and time-consuming than originally expected. The two different cell types grow at different rates, and these rates change as the cells undergo increasing passage number. Cells in culture also have problems of specificity and of losing their differentiated characteristics. However, despite all these problems, we have generated some interesting and potentially very valuable data, indicating how the fetal liver regulates iron transfer across the placenta during pregnancy. The data generated by the co-culture studies is consistent with in vivo studies that

were run in parallel; where we have shown that the fetal liver regulates the maternal metabolism of iron regulating the genes involved in iron metabolism. We plan to confirm that this system also operates in the cell culture system. We will be in a strong position to analyse the processes at a molecular level.

Differential gene expression in placenta; a result of maternal micronutrient deficiencies

A successful partnership was established between EARNEST and the NuGO network this has led to the generation of array results that will be the basis of many years of study for consortium members. By using the whole genome arrays we have been able to identify the vast number of biological processes that are affected by nutritional stress. Many of the identified genes and pathways would never have been on our initial target list, if we were using basic physiological data and theory to establish priorities. This methodology has provided many testable hypotheses for understanding the link between maternal diet and offspring health. Studies are currently underway to verify the array results and map the physiological changes caused by altered target gene expression

Objectively verifiable indicators

Publications

Heaton et al (2008) 'The use of BeWo cells as an in vitro model for placental iron transport' Am J Physiol 295, C1445-54

Meeting Abstracts

Duff et al (2006) 'Liver-placental signalling– development of an *in vitro* model' Winter meeting of the Physiological Society

Richmond et al (2007) 'Identification of gatekeeper genes in nutritional stress using DNA arrays' Biannual meeting of the International Federation of Placental Association

Grants

The data generated in this workpackage was part of pilot data presented in a BBSRC grant application. This joint application between two EARNEST partners, University of Nottingham and the Rowett Institute of Nutrition and Health has been awarded and this will allow us to continue the work begun with the support of the European Union. The NuGO network also awarded a Focus Team grant, "Identification of gatekeeper genes in development" to further study the array data