

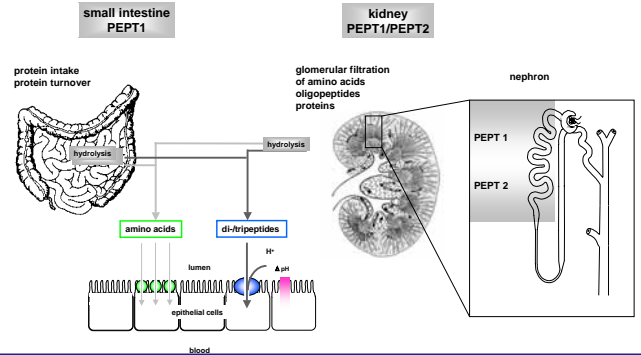
Analysis of differentially expressed genes in kidneys of PEPT2 knockout mice

Isabelle Frey¹, Daniela Sailer, Isabel Rubio-Aliaga², Aleksey Drobyshev², Johannes Beckers² and Hannelore Daniel¹
(1)Molecular Nutrition Unit, Technical University of Munich, Freising, Germany
(2) Institute of Experimental Genetics, GSF,Neuherberg, Germany

Introduction

Transporters for di- and tripeptides that belong to the proton-coupled oligopeptide-transporter family (POT) are found in prokaryotes and eukaryotes. In mammals two different transport systems - PEPT1 and PEPT2 - have been identified. PEPT1 is mainly expressed in the small intestine where it is responsible for the absorption of dietary di- and tripeptides. PEPT2 on the other hand shows a widespread expression within the organism. Its highest expression is found in the brush border membrane of the proximal tubule in the kidney, where it may contribute to the reabsorption of filtered di- and tripeptides.

Cloning and characterization of the murine *Pept2* gene (1) enabled us to generate a knockout mouse line by targeted disruption of the *Pept2* gene. These animals allow the analysis of the physiological role of the peptide transporter PEPT2. Here we provide data on differential gene expression in knockout versus wildtype mice as analysed by a 20K cDNA microarray and qPCR (2,3). To validate that differential gene expression is due to the lack of the peptide transporter PEPT2, kidney mRNA of mice kept on a low, normal or high protein diet (housed in metabolic cages) was analysed by qPCR.



Results I: Identification of differentially expressed genes in kidney tissues of *Pept2*^{-/-} and *Pept2*^{+/+} mice by cDNA microarray analysis

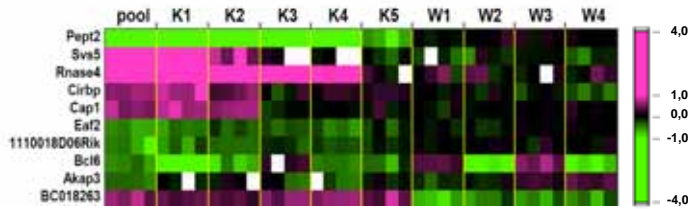


Fig.1 Overview of gene expression profiling data from 40 microarray hybridisations

Results are shown for the ten top differentially expressed genes. Genes are sorted for the minimal absolute log-ratio of the pooled *Pept2*^{-/-} samples ("pool") in descending order. K1 to K5 represent the samples of the five knockout mice and W1 to W4 the samples of four Wildtype mice. K1 to K5 were hybridised versus the pool of all 5 wildtype samples. W1 to W4 were hybridised versus W5. All ten top differentially expressed genes have the same direction of regulation - up (purple) or down (green) in 4 hybridisations of the pooled sample. Some of the genes have different directions of regulation in single knockout or wildtype samples indicating that due to natural variation differential expression in the pooled sample is not reproduced in all individual mice. The colour scale on the right corresponds to the natural logarithm of expression ratio. White colour indicates missing data.

Results III: Differential gene expression under variable feeding conditions

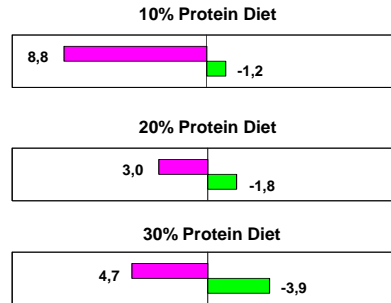


Fig. 2 Expression Ratio of *Rnase4* and *Eaf2* in Knockout versus Wildtype mice on low, normal and high protein diet

■ *Rnase4* ■ *Eaf2*

To validate that differential gene expression is linked to the loss of the peptide transporter PEPT2, kidney mRNA of mice that have been kept either on a low (10%), normal (20%) or high (30%) protein diet was analysed by qPCR with primer pairs for the two genes *Rnase4* and *Eaf2* that were among the ten top ranked genes. For each feeding condition and genotype mRNA of five animals was analysed. Our data show that these genes were regulated under all feeding conditions in the same direction as found in the microarray experiment indicating a link between the loss of PEPT2 and regulation of these genes

Results II: Validation of microarray data by qPCR

Gene	1 st PCR primer pair	2 nd PCR primer pair	Median of log-ratios on cDNA microarrays
<i>Pept2</i>	< -8	< -10	-3,1
<i>Svs5</i>	> 13	> 12	3,3
<i>Rnase4</i>	5,9	4,9	2,1
<i>Cirbp</i>	1,4	1,2	0,85
<i>Cap1</i>	4,5	5	0,77
<i>Eaf2</i>	-1,8	-1,3	-0,85
<i>1110018D06Rik</i>	-1,1	-0,58	-0,85
<i>Bcl6</i>	-1	-1,21	-0,77
<i>Akap3</i>	1	2	-0,58

Table 1: log ratio of qPCR results by two independent primer pairs in comparison to median of log-ratios in 4 microarray hybridisations

For validation of microarray data by qPCR two independent primer pairs were used for each gene. Disagreement between qPCR and microarray data was found only for *Akap3*. This finding is most likely due to crosshybridisation on the microarray as the careful analysis using the „SAFE“-technology (2) indicated unspecific cross hybridisation of this probe due to a 40 nucleotides long GC-rich region

Methods

Animal Maintenance

Mice were maintained at 22 ± 2°C on a 12 hour light:dark cycle. Animals for the microarray experiment had free access to tap water and a standard rodent diet (Altromin 1320) *ad libitum*. Animals for the feeding experiment received a semi synthetic, isocaloric diet that contained either 10%, 20% or 30% protein in metabolic cages for 19 days. Kidneys were collected at age 10–12 weeks and immediately snap frozen in liquid nitrogen.

RNA extraction, cDNA preparation, hybridisation on DNA microarrays and data analysis

Total RNA was isolated with the RNeasy Midi kit (Qiagen) according to the manufacturers protocols. DNA microarray analysis was performed as described in detail in (3). Briefly, 20µg total RNA were used for reverse transcription and indirectly labelled with Cy3 or Cy5 fluorescent dye according to the TIGR protocol. The cDNA was hybridised to a cDNA microarray comprising approximately 20000 clones from the mouse arrayTAG library from Ion Bioscience. For every sample – five *Pept2*^{-/-} versus a wildtype pool, four *Pept2*^{+/+} mice versus the fifth wildtype mouse and a pool of the *Pept2*^{-/-} samples versus the wildtype pool – four independent hybridisations including two dye swaps were performed. Differentially expressed genes were identified by pattern analysis.

Validation of microarray data by qPCR

For ten top ranking genes two independent primer pairs were designed using the Primer3 web interface (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) such that their melting temperatures were in the range of 62-65°C and the differences of melting temperatures within one primer pair was less than 1°C. qPCR was performed on a Light Cycler (Roche) with the FastStart SYBR Green Kit (Roche). Cycle parameters were adjusted individually for each primer pair, however generally the annealing was between 63-66°C for 8-10 seconds, extension at 72°C for 10-12 seconds and melting at 95°C for 15 seconds. Data was analysed by relative quantification with the two housekeeping genes *Hmbs* (hydroxymethylbilane synthase, synonym: PBGD) and *B2m* (beta-2 microglobulin).

qPCR analysis in mice of the feeding experiment.

Total RNA of kidney tissue of five mice per group and genotype was isolated with the Rneasy Mini kit (Qiagen) according to the manufacturers protocols. 1µg of total RNA was transcribed into cDNA with MMLV-RT (Promega) and random hexamers (Fermentas). Primers for *Rnase4* and *Eaf2* were designed by the LightCycler Probe Design software (Roche). qPCR was performed on a LightCycler (Roche) with the FastStart SYBR Green Kit (Roche). Cycle parameters for both primer pairs were annealing at 62°C for 10s, extension at 72°C for 20s and melting at 95°C for 15s. Specificity of PCR products was controlled by melting curve analysis and by agarose gel electrophoresis. Relative quantification was performed with Q-Gene (4) and *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) as housekeeping gene.

Summary

Differential gene expression in kidneys of *Pept2*^{-/-} versus *Pept2*^{+/+} mice was analysed by a 20K cDNA microarray with identification of regulated genes by pattern analysis. Regulation of nine of the ten top ranking genes could be validated by qPCR. To examine whether differential gene expression is due to the lack of the peptide transporter PEPT2, kidney tissue samples of mice that have been fed with diets varying in protein content were analysed by qPCR for the two genes *Rnase4* and *Eaf2*. Under all tested conditions *Rnase4* and *Eaf2* displayed regulation in the same direction as in the microarray experiment. Our data show that cDNA microarray screening in combination with qPCR is a useful tool in identification of differentially expressed genes here used for the characterization of compensatory gene regulation in knockout animals.

References

- Rubio-Aliaga I, Boll M, Daniel H: Cloning and characterization of the gene encoding the mouse peptide transporter PEPT2, *Biochem Biophys Res Commun* 276, 734-741, 2000
- Drobyshev AL, Machka C, Horsch M, Seltmann M, Liebscher V, Hrabá de Angelis M, Beckers J: Specificity assessment from fractionation experiments (SAFE): a novel method to evaluate microarray probe specificity based on hybridisation stringencies, *Nucleic Acids Res* 31, E1-1(2003)
- Drobyshev AL, Liebscher HV, Horsch M, Frey I, Rubio-Aliaga I, Adamsky J, Daniel H, Hrabá de Angelis M, Beckers J: Identification of regulated Genes in DNA microarray studies considering natural variation in gene expression levels, submitted
- Muller PY, Janovjak H, Miserez AR, Dobbie Z: Processing of gene expression data generated by quantitative real-time RT-PCR, *BioTechniques* 32 (6), 1372-1378 (2002)