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Effect of zinc deficiency on the mRNA expression pattern in liver and jejunum of adult rats: Monitoring gene expression using cDNA microarrays combined with real-time RT-PCR

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Abstract

In the study presented here, the effect of zinc deficiency on mRNA expression levels in liver and jejunum of adult rats was analyzed. Feed intake was restricted to 8 g/day. The semi-synthetic diet was fortified with pure phytate and contained either 2 μg Zn/g (Zn deficiency, $n = 6$) or 58 μg Zn/g (control, $n = 7$). After 29 days of Zn depletion feeding, entire jejunum and liver were retrieved and total RNA was extracted. Tissue specific expression pattern were screened and quantified by microarray analysis and verified individually via real-time RT-PCR. A relative quantification was performed with the newly developed *Relative Expression Software Tool* © on numerous candidate genes which showed a differential expression.

This study provides the first comparative view of gene expression regulation and fully quantitative expression analysis of 35 candidate genes in a non-growing Zn deficient adult rat model. The expression results indicate the existence of individual expression pattern in liver and jejunum and their tissue specific regulation under Zn deficiency. In addition, in jejunum a number of B-cell related genes could be demonstrated to be suppressed at Zn deficiency. In liver, metallothionein subtype 1 and 2 (MT-1 and MT-2) genes could be shown to be dramatically repressed and therefore represent putative markers for Zn deficiency. Expression results imply that some genes are expressed constitutively, whereas others are highly regulated in tissues responsible for Zn homeostasis.

Keywords: Zinc deficiency; Gene expression pattern; cDNA microarray; Real-time RT-PCR; Relative expression; Adult rats; Metallothionein

1. Introduction

Zinc (Zn) is an essential metal involved in a variety of biological functions [1,2]. Its deficiency is associated with a wide range of physiological defects including the neurological, immune and reproductive system as well as disorders of the skin [3]. The importance of Zn in cell physiology is related mainly to its intracellular involvement into enzyme catalysis, protein structure, protein-protein interactions, and protein oligonucleotide interactions [4]. The intracellular accumulation of Zn is a sum of influx and efflux processes via Zn transporter proteins, like divalent cation transporter 1 and the four Zn transporter proteins ZnT1 to ZnT4 [5,6]. Intracellular Zn is bound to metallothionein (MT), which is one of the strongest biological binding ligands for Zn and regulates the intracellular free Zn levels through intracellular binding [7]. MT gene expression is regulated by cellular Zn concentrations and a close correlation between Zn and MT mRNA expression in liver and pancreas is well documented [8,9]. Homeostasis regulates Zn concentration in cells and tissues quite efficient and prevents the organism from excessive accumulation over a wide range of dietary Zn intake [10–12]. Therefore, Zn is virtually non-toxic to the living organisms [13].

Up to date the general knowledge of changes in mRNA expression pattern derived from Zn deficiency is limited to array experiments in small intestine and kidney of growing rats [14,15] and T-lymphocytes from murine thymus in growing mice [16]. In addition some semi-quantitative ex-

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Table 1
Primer sequences for real-time RT-PCR

Candidate genes	Forward Primer	Reverse Primer
Agrin: agrin precursor.	CTGCAGAGCAACCACTTTG	CCAAGCCACAGGGCTCCATC
AMBP: AMBP protein precursor	GAGGTGAATGTGTCCCTGG	GACTATGGGGAGATTGCAGG
Beta-actin	CGACAGCAGTTGGTTGGAGC	GGTCTCAAGTCAGTGTACAG
BCL1: G1/S-specific cyclin D1 (BCL-1 oncogene).	CTGGTGAACAAGCTCAAGTG	GGCGCAGGCTTGACTCCAG
CD19: B-lymphocyte antigen CD19 precursor	TTCTATGAGAACGACTCCAAC	TCCTCTCATATCCTCATAGGAC
CD22: B-cell receptor CD22 precursor	AGGAGGCTGCGTGTGTCCAT	TAGTAGACAGTAAGGGTGCTG
CD36: platelet glycoprotein IV (GPIV) (CD36 antigen)	CATCATATGGTGTGCTAGACA	CTCATCACCAATGGTCCCAG
CD37: leukocyte antigen CD37	CTGCGCTGCTGCGGCTGGC	TTGTTGTGCAGCCACTTCTG
CD53: leukocyte surface antigen CD53	TCCTCCTTGCTGAGGTGACC	AGGGTCAGTGCAAAGGACAT
CD72: B-cell differentiation antigen CD72	ATCTGCAGGTGTCTCAGCAG	GGACAGCAGGTGTCTGCTGA
CD79B: B-cell antigen rec. comp. associated protein beta-chain prec.	AGGACAATGGCATCTACTTCTG	TCATAGGTGGCTGTCTGGTC
CDH1: epithelial-cadherin precursor	CACTGCCAACTGGCTGGAG	GGGTTAGCTCAGCAGTAAAG
CIRBP: cold-inducible RNA-binding protein	GCAGATCCGAGTAGACCAG	CTCTGGAGCCTCCGTAGCC
CR2: complement receptor type 2 precursor (CD21 antigen)	AGTCCCCAGAGCCAGTGCCA	GATACTTCTCGTGCTTCTAAAT
CYP2B1/2: Cytochrome P450 2B1	GAGCTCTGTGAATGGCACTGAAGAAG	AGTCTGTAGACATAGCACTG
CYP7A1: Cytochrome P450 7A1	GAGCTCATGCATGCCAATGAGAAGAG	AATTCCACATACCTCAGAGC
DBP: D-site-binding protein	GGGACACATTTGTCCGAGCC	GCAGAGTTGCCTTGCGCTCC
DUSP1: dual specificity protein phosphatase 1	GCTGGTCCTTATTTATTTAAC	AATACTGGTAGGTATGTCAAGC
ES-4: liver carboxylesterase 4 (and 5) precursor	CACAGTATGACCAGAAAGAAG	TTTCTCTTTCCTGGGTTCTC
FMO3: dimethylaniline monooxygenase 3	GAGCTCGTCACCGACAATAACCAGAC	AGGAGAAATGACTTATGCTC
HP-1:Haptoglobin-1 precursor	GGAGGAGGACACCTGGTATG	ATTGACTCAGCAATGCAGGG
HSP105: Heat-shock protein 105 KDA	CTGCAGCATTATGCCAAGAT	TTTGGGCCATTTGGAGTTCT
IL2RG: Cytokine receptor common gama chain precursor (CD132 antigen)	TCTCCTTGCCTAGTGTGGATG	GTCTGGCTGCAGACTCTCAG
IL-6R-Beta: interleukin-6 receptor beta chain precursor (CD130 antigen)	TTGCCCAGTGGTCACCTCAC	AGATCTTCTGGCCGCTCCTC
MT-1: Metallothionein-1	TGGACCCCAACTGCTCCTG	TCAGGCACAGCACGTGCAC
MT-2: Metallothionein-2	TGGACCCCAACTGCTCCTG	TCAGGCGCAGCAGCTGCAC
NRF1: Nuclear factor erythroid 2 related factor 1	ACAGATGAAGCAGAAGGTCC	GCCCTTCTCCAGTTTCGGTC
PAP3: Pancreatitis-associated protein 3 precursor	TCTGGAAGTCACTGTGGGAC	TCTCAGGCCACAGTACACAC
RORC: Nuclear receptor ROR-gamma	CATCTCTGCAAGACTCATCG	CCTTCCTCCAGATCACTTTG
RXRA: Retinoic acid receptor RXR-alpha	CTTATGGGCCCAAAAGATGC	CCAACTCCTGCAGCTCCAGG
SMPD2: Sphingomyelin phosphodiesterase 2	CTGTTGTGTGGAGACCTCAA	GGGTCAAAGCCTGTAGTGGT
STAT5B: Signal transducer and activaytor of transcription 5B	GAAGACACGATGGACGTGGC	CTAGTGCCACTATGCACAG
SULT1AX: Thermostable ST1A/STM families of phenol sulfotransferases	GAGCTCCCACTGAGATTATGGACCAC	ATGGGCTGTGTCAGTTTGCC
TF: Serotransferrin precursor (siderophilin)	AGGAGCAGAGTACTTGCAAGC	AAGACGGACACAGTTAGCCC
TTR: Transthyretin precursor (prealbumin)	TTCACAGCCAATGACTCTGG	TCTTCCCGAGTTGCTAACAC

pression studies were performed on basis of Northern-Blot analysis [5] densitometric analysis of RT-PCR [17,18], or competitive RT-PCR [19,20]. No information on the mRNA expression pattern in adult and non-growing individuals is available.

Therefore, it was the aim of this study to elucidate the effect of Zn deficiency on the expression pattern in the Zn absorbing tissues jejunum as well as in liver. The respective tissues were retrieved from a rat experiment [21], which represented a newly established animal model on Zn deficiency in adult individuals [22,23]. Published Zn deficiency models are usually performed with fast-growing rats. But the intensive anabolic situation produces severe interactions between Zn deficiency *per se* and the metabolism *in vivo*. Respective results may thus not fully reflect the situation in adults. To overcome this methodological disadvantage, an animal model to study Zn deficiency in adult non-growing rats was developed [21]. In a knowledge-driven approach we have carefully selected known, toxicologically relevant cDNAs in order to find potentially new marker genes which are regulated under Zn deficiency. A rat specific PIQOR™ array was applied consisting of approximately 1000 cDNAs. (for a full gene list see www.memorec.com/research and development/publications expression profiling/ download supplementary material: gene list). The PIQOR™ system allows the parallel identification and quantification of thousand transcripts from two different samples (e.g., diseased vs. normal tissue or samples of physiologically vs. un-physiologically treated animals) [25–29]. For verification of candidate genes found in array experiments, quantitative reverse transcription - polymerase chain reaction (RT-PCR) on a real-time platform represents a suitable tool. Thus during the recent years, real-time RT-PCR using SYBR Green I technology is more and more used to quantify physiologically changes in gene expression [30] and to verify gene expression results derived from microarrays [31,32].

Table 2
Candidate genes in liver with more than 2-fold regulation on microarray experiments (each gene n = 4) were verified via real-time RT-PCR (n = 6/7). Significance levels between control and Zn deficient group determined by randomisation test are indicated as followed: ** $p < 0.01$; *** $p < 0.001$

Regulated gene in liver under zinc deficiency	UniGene Accession number	Microarray		Real-time RT-PCR		
		x-fold	variation	x-fold	x-fold (normalised)	variation
Interleukin-6 receptor beta chain precursor (IL-6R-Beta) (CD 130 Antigen)	Rn.12138	+2.32	4%	+1.64	+1.38	2%
Dual specificity protein phosphatase 1 (DUSP1)	Rn.31120	+2.28	4%	+1.20	+1.02	4%
Thermostable ST1A /STM families of phenol sulfotransferases (SULT1AX)	Rn.1507	+2.19	4%	+1.49	+1.25	2%
Dimethylaniline monooxygenase 3 (FMO3)	Rn.11676	+2.01	7%	+1.43	+1.20	2%
Metallothionein-II (MT-2)	Rn.2714	−25.00	2%	−55.78***	−66.46***	6%
Metallothionein-I (MT-1)	Rn.2714	−20.00	4%	−36.82**	−43.85**	14%
D-site-binding protein (Albumin D box-binding protein) (DBP)	Rn.11274	−3.33	9%	−95.01***	−113.13***	9%
G1/S-specific cyclin D1 (BCL-1 oncogene) (BCL1)	Rn.9471	−3.33	8%	−1.32	−1.58	5%
Sphingomyelin phosphodiesterase 2 (SMPD2)	Rn.18572	−2.94	−%	−1.15	−1.37	2%
Sodium- and chloride-dependent glycine transporter 1 (GLYT-1)	Rn.32110	−2.70	6%	n.d.	n.d.	n.d.
Agrin precursor (AGRN)	Rn.2163	−2.44	21%	+1.08	−1.10	1%
Cytochrome P450 7A1 (CYP7A1)	Rn.10737	−2.27	9%	−1.08	−1.28	4%
Retinoic acid receptor RXR-ALPHA (RXRA)	Rn.34870	−2.13	1%	−1.05	−1.25	1%
Signal transducer and activator of transcription 5B (STAT5B)	Rn.54486	−2.08	−%	−1.35	−1.61	5%
Cold-inducible RNA−binding protein (CIRBP)	Rn.28931	−2.08	7%	−1.72	−2.05	5%
Nuclear factor erythroid 2 related factor 1 (NRF1)	Rn.21931	−2.08	10%	−1.28	−1.52	4%
J Domain containing protein 1 isoform A (JDP1)	Rn.9583	−2.08	5%	n.d.	n.d.	n.d.
Hepatocyte nuclear factor 3-gamma (HNF-3G)	Rn.10949	−2.00	12%	n.d.	n.d.	n.d.

n.d. = real-time RT-PCR was not performed

2. Material and methods

2.1. Animal experiment

The rat tissues were retrieved from an animal model described earlier [21]: 13 adult female, non-growing rats (average weight 212 g) were fed a purified, phytate enriched diet at restricted amounts covering all necessary feed components for maintenance (8.0 g per head and day). Dietary Zn remained for the Zn deficiency group ($n = 6$) at its native level of 2 μg/g or was supplemented in control group with additional $ZnSO_4$ at amounts covering the requirement of Zn at 58 μg/g ($n = 6$). Both feeding regimen differed only in the Zn concentration, all remaining feed components were identical. Rats suffering from Zn deficiency were euthanised after 22 or 29 days (*n* 6). Rat liver (central section, blood vessel free) and the middle part of the jejunum (including all tissue layers) were removed immediately after euthanising, cleaned with physiological salt buffers (0.9% NaCl), snap frozen in liquid nitrogen and stored −80°C until total RNA extraction.

2.2. Total RNA extraction

The total RNA extraction was performed with Trizol (Roche Diagnostics, Mannheim, Germany) according to the manufactures instructions. From extracted total RNA the mRNA was isolated by Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). Qualitative integrity test of purified total RNA, mRNA and quantitative measurements were done with capillary electrophoresis using a Bioanalyser 2100 (Agilent Technologies, Palo Alto, California, USA).

2.3. cDNA array production

Defined 200 to 400 bp fragments of selected cDNAs were generated by RT-PCR using Superscript II (Invitrogen, Groningen, The Netherlands) and sequence-specific primers and RNA derived from appropriate rat tissue and cell lines. A list of all genes including their Unigene accession numbers (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) is available in the Tables 2 and 3. Each fragment was cloned into pGEM®-T Vector (Promega, Mannheim, Germany) and sequence-verified. Correct annotation of the genes was verified by automatic blast search using the Unigene and SwissProt databases. Inserts were amplified (Taq PCR Master Mix, Qiagen) using vector-sequence-derived primers with the sense primer carrying an 5′-amino-modification. PCR fragments were purified (QIAquick 96 PCR BioRobot Kit, Qiagen), checked on an agarose gel and diluted to a concentration of 100 ng/μl. Amplified inserts were transferred to a 384-well plate and spotted 4 times each on treated glass slides with a customised ink jet spotter. Each probe was spotted by dispensing 6 drops of 100 pl each. Probes were re-hydrated 2 h in a humidified chamber and blocked [29,33,34]. Therefore in the array experiment 4 identical repeats were present (Fig. 1).

2.4. Sample labeling and hybridization

For the array experiments the total RNA of the control group ($n = 7$ rats) and the Zn deficiency group ($n = 6$ rats) were pooled separately in identical concentrations for anal-

Table 3
Candidate genes in jejunum with more than 2-fold regulation on microarray experiments (each gene n = 4) were verified via real-time RT-PCR (n = 6/7). Significance levels between control and Zn deficient group determined by randomisation test are indicated as followed: ** $p < 0.01$; A trend of regulation is indicated by (*) $0.05 < p < 0.10$.

Regulated gene in jejunum under zinc deficiency	UniGene Accession number	Microarray		Real-time RT-PCR		
		x-fold	variation	x-fold	x-fold (normalised)	variation
Cytochrome P450 2B1 (CYP2B1)	Rn.2287	+2.46	2%	+2.93(*)	+3.27	7%
Epithelial-cadherin precursor (E-CADHERIN) (CDH1)	Rn.1303	+2.33	5%	+3.79(*)	+4.18	7%
Heat-shock protein 105 KDA (HSP105)	Mm.34828	+2.16	5%	+4.42(*)	+4.94(*)	11%
Platelet glycoprotein IV (GPIV) (CD36 ANTIGEN)	Rn.3790	+2.05	4%	+1.09	+1.36	4%
Nuclear receptor ROR-GAMMA (RORC)	Mm.4372	+2.01	4%	+3.45(*)	+3.85(*)	9%
Transthyretin precursor (Prealbumin) (TTR)	Rn.1404	−5.56	− %	−3.52 **	−3.15 **	3%
Pancreatitis-associated protein 3 precursor (PAP3)	Rn.9729	−3.85	3%	+1.56	+1.79	18%
Haptoglobin-1 precursor (HP1)	Rn.10950	−3.57	7%	−2.32	−2.08	5%
Serotransferrin precursor (Siderophilin) (TF)	Rn.2514	−3.57	7%	−4.08	−3.33	9%
B-lymphocyte antigen CD19 precursor (CD19)	Mm.4360	−3.45	6%	−17.87	−15.96	48%
AMBP protein precursor (AMBP)	Rn.18721	−3.33	− %	−1.76	−1.58	2%
B-cell antigen receptor complex associated protein beta-chain precursor (CD79B)	Rn.44358	−3.13	6%	+1.09	+1.22	3%
D-site-binding protein (DBP)	Rn.11274	−2.94	6%	−1.04	+1.08	3%
B-cell receptor CD22 precursor (CD22)	Mm.1708	−2.70	− %	+1.23	+1.38	2%
Leukocyte antigen CD37 (CD37).	Rn.2357	−2.70	10%	+1.28	+1.43	6%
B-cell differentiation antigen CD72 (CD72)	Mm.88200	−2.70	4%	−1.54	−1.37	5%
Leukocyte surface antigen CD53 (CD53)	Rn.31988	−2.63	10%	+4.73	+5.27	23%
CCR4 C X C chemokine receptor type 4 (CXC R4)	Rn.44431	−2.44	2%	n.d.	n.d.	n.d.
Complement receptor type 2 precursor (CR2) (CD21 antigen)	Hs.73792	−2.33	4%	+1.31(*)	+1.46(*)	11%
B lymphocyte chemoattractant precursor (B cell-attracting chemokine 1) (BCA-1)	—	−2.27	8%	n.d.	n.d.	n.d.
Liver carboxylesterase 4 (and 5) precursor (ES-4 / ES-5)	Rn.34885	−2.22	8%	+1.45	+1.78	5%
Protein kinase C beta-I and beta-II type (PRKCB)	Rn.9745	−2.22	10%	n.d.	n.d.	n.d.
Interleukin-2 receptor gamma chain (IL2RG) (CD132 antigen)	Rn.14508	−2.13	2%	+1.08	+1.32	14%
Ras-related C3 botulinum toxin substrate 2 (RAC2)	Rn.2863	−2.08	8%	n.d.	n.d.	n.d.

n.d. = real-time RT-PCR was not performed

ysis. 100 μg of pooled total RNA each was combined with a control RNA consisting of an in vitro transcribed *E. coli* genomic DNA fragment carrying a 30 nt poly(A)$^+$-tail and the mRNA was isolated (Oligotex mRNA Mini Kit, Qiagen). The resulting mRNA was diluted to 17 μl and combined with 2 μl of a second control RNA, a mixture of 3 different transcripts. The mRNA was then reverse-transcribed by adding it to a mix consisting of 8 μl 5×First Strand Buffer (Invitrogen), 2 μl Primer-Mix (oligo-dT and randomeres) (memorec), 2 μl low C dNTPs (10 mM dATP, 10 mM dGTP, 10 mM dTTP; 4 mM dCTP), 2 μl FluoroLink Cy3/5-dCTP (Amersham Pharmacia Biotech, Freiburg, Germany), 4 μl 0.1 M DTT and 1 μl RNasin (20 to 40 U) (Promega). 200 U of Super Script II Reverse Transcriptase (Invitrogen) was added, incubated at 42°C for 30 min followed by the addition of 1 μl of Super Script II Reverse Transcriptase (Invitrogen) and incubated under the same conditions as detailed above. 0.5 μl of RNaseH (Invitrogen) was added and incubated at 37°C for 20 min to hydrolyze RNA. Cy3- and Cy5-labeled samples were combined and cleaned up using QIAquick™ (Qiagen). Eluents were diluted to a volume of 50 μl. Fifty μl of 2× hybridization solution (memorec) pre-warmed to 42°C was added. Hybridization was performed according to manufacturer's guidelines (memorec) using a GeneTAC hybridization station (Perkin Elmer, Langen, Germany). In brief: Slides were fixed in the GeneTAC hybridization station. 100 μl of prehybridisation solution were added and slides were prehybridized at 65°C for 30 min. Thereafter, 100 μl purified, mixed Cy3 and Cy5 labeled probes in 2× hybridization solution were pipetted onto the slides thereby displacing the prehybridisation solution. Hybridization is then performed for 14 h at 65°C, followed by 4 washing steps carried out at 50°C (see also instruction manual, memorec).

2.5. Data analysis of array hybridizations

Microarray experiments were performed according to the MIAME guidelines [35]. Image capture and signal quantification of hybridized PIQOR™ cDNA arrays were done with the ScanArray3000 (GSI Lumonics, Watertown, MA) and ImaGene software version 4.1 (BioDiscovery, Los Angeles, CA). Scanning was performed twice per slide. The first low resolution scan (50 μm) was performed in order to localize the spot with the brightest signal intensity on the array. Via an autocalibration feature implemented in the

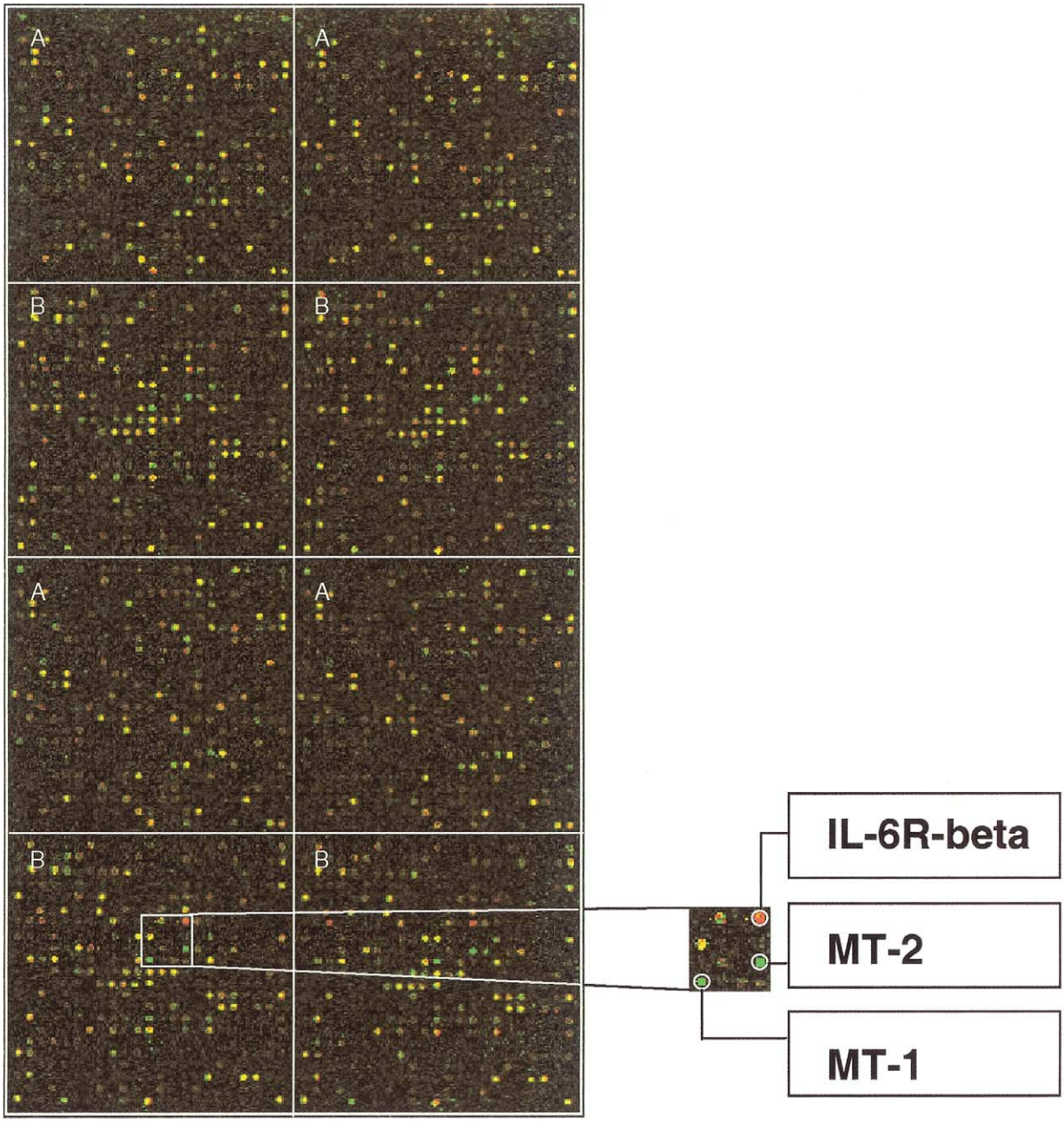

Fig. 1. Representative example of a gene expression pattern captured as an image of a cDNA-array hybridized with Cy3-labeled control sample (green) and Cy5-labeled sample (Zn deficiency in red). Each of the 1001 cDNAs were spotted either in quadrant A and B. Four replicates for each cDNAs were spotted, resulting in four A and B quadrants, respectively. A magnification for the most up-regulated (MT-1 and MT-2) and down-regulated (IL-6R-beta) genes is shown.

Scanner software, optimal laser power and photo multiplier tube parameters were calculated. The second high resolution scan (10 μm) was performed using these optimized scanning parameters thereby preventing the generation of oversaturated signals. For each spot, the local signal was measured inside a circle adjusted to the individual spot (160 to 230 μm diameter), and background was measured outside the circle within specified rings 30 μm distant to the signal and 100 μm wide. Signal and background was taken to be the average of pixels between defined low and high percentages of maximum intensity with percentage parameter settings for low/high being 0/97% for signal and 2/97% for background. Local background was subtracted from the signal to obtain the net signal intensity and the ratio of Cy5/Cy3. Subsequently, the mean of the ratios of 4 corresponding spots representing the same cDNA was computed. The mean ratios were normalized to the median of all mean ratios by using only those spots for which the fluorescent

intensity in 1 of the 2 channels was two times the negative control. The negative control for each array was computed as the mean of the signal intensity of 4 spots representing salmon sperm and 4 spots representing spotting buffer only. Only genes displaying a net signal intensity 2-fold higher in the control or treatment sample than in the negative control were used for further analysis.

3. Reverse transcription for real-time PCR

One μg of purified total RNA from each individual rat tissue preparation ($n = 13$) were reverse transcribed with 100 U of M-MLV Reverse Transcriptase RNase H^- Point Mutant Reverse Transcriptase (Promega) using 100 μM random hexamer primers (Promega) according to the manufactures protocol.

3.1. Primer

Primers used for real-time RT-PCR were identical to such used for the generation of arrayed cDNAs (memorec). 35 candidate gene primer pairs were designed (Table 1) such that the corresponding amplified cDNA fragment fulfils several selection criteria with respect to e.g., homology to other known genes (<85%) and uniformity of the fragment length. Selection criteria are summarized in detail elsewhere [34].

3.2. Real-time PCR

For each investigated transcript a master-mix of the following reaction components was prepared to the indicated end-concentration: 6.4 μl water, 1.2 μl $MgCl_2$ (4 mM), 0.2 μl forward Primer (0.4 μM), 0.2 μl reverse Primer (0.4 μM) and 1.0 μl LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics). Nine μl of master-mix was filled in the glass capillaries and 1 μl volume, containing 25 ng reverse transcribed total RNA, was added as PCR template. Capillaries were closed, centrifuged and placed into the LightCycler rotor. The following real-time PCR protocol was used for all genes: *denaturation program* (10 min @ 95°C), *amplification and quantification program* repeated 40 times (15 s @ 95°C; 10 s @ 60°C; 30 s @ 72°C with a single fluorescence measurement), *melting curve program* (60°C to 99°C with a heating rate of 0.1°C/s and continuous fluorescence measurements), *cooling program* down to 40°C.

3.3. Relative quantification

For the described relative quantification an appropriate mathematical model is necessary. Herein the *"delta-delta CP method"* for comparing relative expression results between treatments in real-time PCR was applied as described earlier [36,37].

$$R = 2^{-[\Delta CP\ \text{sample} - \Delta CP\ \text{control}]} \quad (1)$$

$$R = 2^{-\Delta\Delta CP} \quad (2)$$

Therefore the determination of crossing points (CP) for each transcript is essential. The CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. In this study *"second derivate maximum method"* was performed for CP determination, using LightCycler Software 3.5 [38]. The relative expression ratio of a target gene is computed, based on mean real-time PCR CP deviation (ΔCP) of a unknown sample group vs. the control group [39]. The *"delta-delta CP method"* presumes an optimal and identical real-time amplification efficiencies of target genes (herein the candidate genes summarized in Table 1) and reference gene (herein beta-actin) of $\mathbf{E_{target\ gene} = E_{reference\ gene} = 2}$.

$$R = \frac{(E_{target})^{\Delta CP\text{target}\ (MEAN\ control - MEAN\ sample)}}{(E_{ref})^{\Delta CP\text{ref}\ (MEAN\ control - MEAN\ sample)}} \quad (3)$$

$$R = \frac{2^{\Delta CP\ \text{candidate gene}\ (MEAN\ control - MEAN\ sample)}}{2^{\Delta CP\ \text{beta-actin}\ (MEAN\ control - MEAN\ sample)}} \quad (4)$$

3.4. Statistical analysis

For both groups the expression ratio results of the investigated transcripts are tested for significances by a randomisation test. The *Pair Wise Fixed Reallocation Randomisation Test* © is running within the *Relative Expression Software Tool*© (REST©) which was developed in order for a better understanding and easier calculation of relative quantification analysis in real-time RT-PCR [39]. Expression results can be either normalized according to a housekeeping gene (reference gene expression) or not normalized, as wanted by the software user. The latest versions of REST© and REST-XL© and examples for the correct use can be downloaded at the URL: http://www.wzw.tum.de/gene-quantification/

4. Results

4.1. Total RNA and mRNA concentrations

Extracted total RNA concentrations in liver and jejunum were constant with respect to both tissues and treatments [average RNA concentrations: liver control group (1534 ± 713 ng/mg tissue), Zn deficiency group (1321 ± 370 ng/mg tissue), jejunum control group (1970 ± 477 ng/mg tissue), Zn deficiency group (2569 ± 712 ng/mg tissue)]. Liver and jejunum purified mRNA integrity was verified additionally by capillary electrophoresis (Bioanalyser 2100, Agilent Technologies, data not shown).

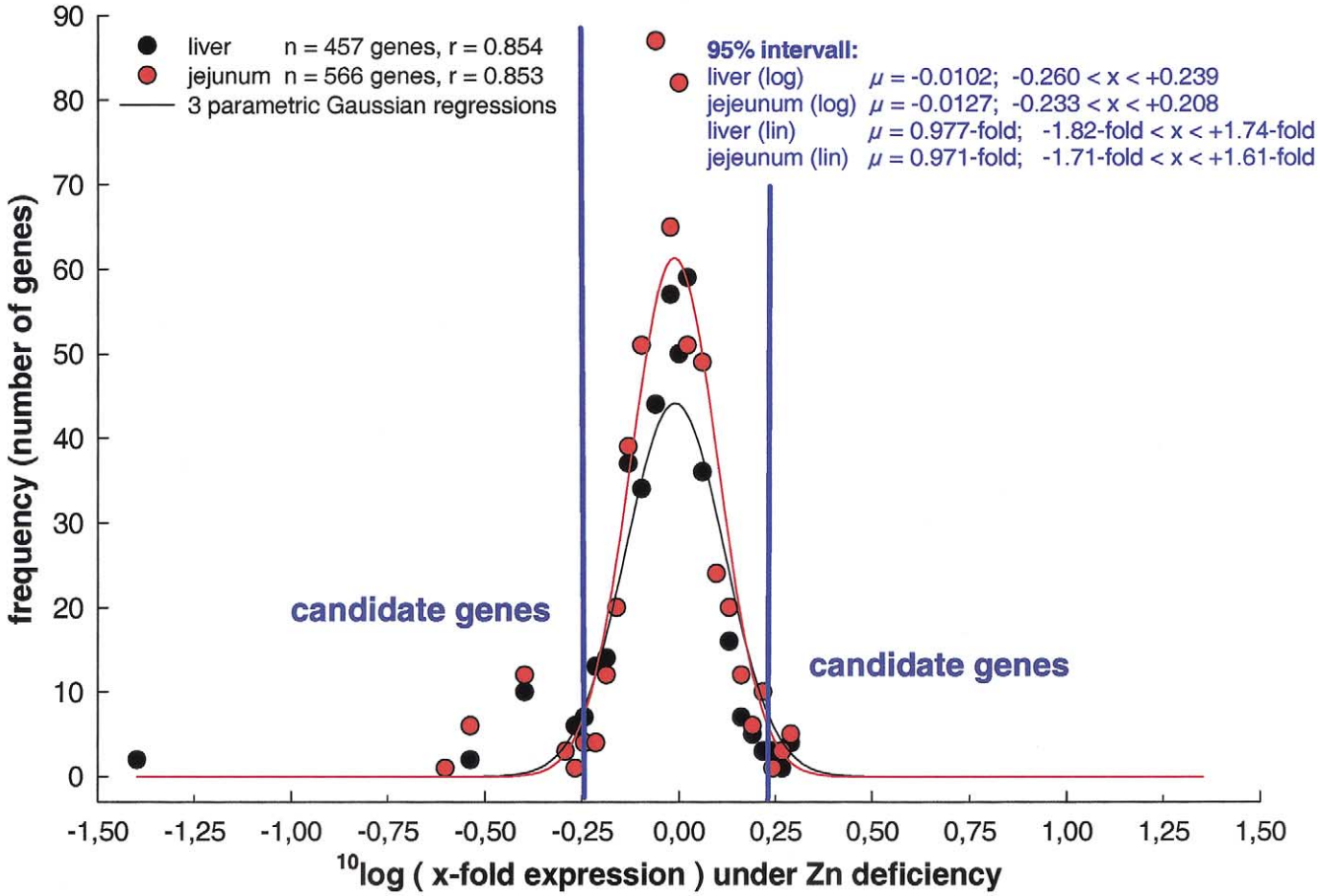

Fig. 2. Frequency and level of down- or up-regulation of regulated genes of microarray experiments in liver and jejunum of Zn deficiency rats. Frequency plot of both tissue expression pattern exhibit a three parametric Gaussian distribution ($P < 0.0001$). Mean (μ) and boarders of confidential interval are indicated ($\mu \pm 1.96$ times the standard deviation of the Gaussian distribution). Significant different expressed genes ($P < 0.05$) were selected outside the 95% confidential interval. Lines indicate an approximation of 95% interval in liver and jejunum.

4.2. Microarray analysis

RNAs from liver and jejunum of control samples were reverse transcribed and Cy3 labeled, RNAs from Zn deficiency samples were reverse transcribed and Cy5 labeled. A false color overlay from the hybridization of liver samples on a PIQOR™ array is shown in Fig. 1. Spots of the strongest down-regulated genes MT-1 and MT-2 as well as up-regulated gene IL-6R-beta are indicated (Fig. 1).

4.3. Candidate genes

From the available 1001 genes present on the microarray 457 genes in liver (45.7%) and 566 genes in jejunum (56.6%) were found to be expressed with signal intensities in at least one of the two channels for Cy3 or Cy5 greater than two-fold above negative controls. Genes fulfilling this stringent criteria were included for further analysis. The frequency of gene regulation of both tissues are shown in Fig. 2. Genes were combined in classes (0.1-fold expression width), between 1-fold and 2-fold expression ratio and over 2-fold expression in wider ranges. A three parametric Gaussian regression was calculated for each tissue on the bases of a logarithmical conversion (^{10} log) of the median expression ratios of each class. This resulted in high regression coefficient ($r_{liver} = 0.854$, $P < 0.0001$; $r_{jejunum} = 0.853$, $P < 0.0001$) and in a normal distribution of both frequency datasets. A 95% confidential interval was calculated for the x-fold expression ratio (x), two sided from the mean (μ) according to the Gaussian distribution **(μ − 1.96 × standard deviation < μ < μ + 1.96 × standard deviation)** and the two sided 2.5% significance level borders were defined ($P < 0.05$). For liver and jejunum the following 95% confidence intervals were calculated: **−1.82-fold < x_{liver} < +1.74-fold, −1.71-fold < $x_{jejunum}$ < +1.61-fold**, respectively.

According to the derived cut offs 85 candidate genes were selected in total (Fig. 2). In liver 10 genes were up-regulated higher than 1.74-fold. The mean variation of the calculated expression ratio was 6.3%, calculated from the signal ratios of 4 repeats of cDNA spots of each gene per array experiment. In liver 23 genes were down-regulated

more than 1.82-fold with a variation of 8.6%. In jejunum 25 genes were up-regulated higher than 1.61-fold under zinc deficiency (variation = 4.9%), and 27 genes were down-regulated 1.71-fold with an average variation of 7.1%.

4.4. Confirmation of primer and RT-PCR product specificity

Some of the candidate genes in liver and jejunum with a significant regulation on microarray experiments were verified via real-time RT-PCR. Therefore a list of 15 candidate genes in liver and 20 candidate genes jejunum are shown in Table 2 and 3. Specificity of the desired RT-PCR products were documented with high resolution gel electrophoresis and by melting curve analysis [38]. The product specific melting curves showed only single peaks and no primer-dimer peaks or artifacts.

4.5. Expression of housekeeping gene (reference gene)

Beta-actin was used as housekeeping gene and reference gene in order to compare the quantified mRNA molecules of the various candidate genes in the relative expression ratio model (equation 4). The real-time RT-PCR efficiency was set for all factors identically to 2 **(E = 2.0).** The beta-actin expression showed no significant regulation under Zn deficiency in liver and jejunum as demonstrated in the microarray analysis (-1.12 in liver; +1.02 in jejunum) and real time RT-PCR (+1.19 in liver; −1.11 in jejunum).

4.6. Relative changes in mRNA expression due to Zn deficiency

In liver the expression profile found in the microarray experiments could be confirmed, except in one case for the agrin receptor (AGRN), by real-time RT-PCR for the candidate genes. The trends of regulation (up- or down-regulation) calculated by REST© were similar in absolute and in normalized expression ratio results. High significant different expression levels were found for MT-1, MT-2 and D-site binding protein (Table 2). In jejunum the trend of up-regulation under Zn deficiency could be confirmed for all 5 candidate genes (Table 3). Four of these expression levels tend to be significant ($0.05 < P < 0.10$). For 6 out of the proposed 15 down-regulated genes in jejunum array experiments, the trend of regulation could be confirmed. Nine slightly down-regulated candidate genes by microarray analysis were shown to be not regulated or only slightly up-regulated by real time PCR, but none of them significantly.

Variations of expression levels differ between used quantification methods and platforms. The array exhibit a mean intra-assay variation of candidate genes of 6.7% (n = 85) within the four repeats and the kinetic RT-PCR on the LightCycler showed an average variation of 7.5% (n = 35).

5. Discussion

Goal of the study was to establish a broad and sensitive system to verify gene expression of Zn regulated genes as well as to verify and quantify them in liver and jejunum. Herein a cDNA array was used to find a panel of 85 Zn sensitive candidate genes and kinetic RT-PCR was performed to confirm 35 of the differential expressed genes identified by microarray experiments.

5.1. Methodical considerations

Microarray based screening of tissue specific gene expression and confirmation of putative candidate target genes by kinetic RT-PCR represents a powerful combination. Hereby the advantages of both quantification systems were added - a high throughput of the microarray platform as well as sensitivity and specificity of the real-time RT-PCR platform [30,40,41].

In the microarray experiments hybridized with liver mRNA, 33 out of 457 genes or jejunum mRNA 52 out of 566 genes were found to be significant differentially regulated ($P < 0.05$). From these 85 differentially regulated genes, 35 were included in further analysis (Table 2 and 3). As presented, we have designed and validated several real-time RT-PCR assays to verify the candidate genes on LightCycler platform. Kinetic RT-PCR amplification of candidate genes was shown to be sensitive, with high precision and reproducibility. In liver all genes could be confirmed in their general expression pattern, either up- or down regulated. In jejunum 6 out of 15 down regulated genes found in the microarray analysis could be confirmed by RT-PCR. The remaining 9 genes showed either no or only a slight up-regulation, non of them significantly.

The observed differences of single gene expression levels derived from array experiments and kinetic RT-PCR experiments are at least in part due to different methodological processing and handling of the samples. In array experiments extracted RNA samples were group wise pooled in identical concentrations for analysis (n = 2 pool from 6 or 7 animals) and therefore the result is a weighted result of the highest mRNA concentration present in the pool. On the other hand, for the group wise comparison performed by REST© (n = 6/7 samples) the mean CP of all sample was calculated and samples were considered equally in the expression ratio calculation as in the statistical model (randomisation test). Further variations of expression ratios can occur from the normalization procedures performed to standardize the tissue individual expression levels of RNA extraction efficiencies. Normalization of the array expression results were totally different from the normalization done for kinetic RT-PCR. On the microarray the fluorescence levels of both channels (Cy3 and Cy5) were normalized to the median of all ratios by using only those spots for which the fluorescent intensity was 2 times the negative control [29]. In real-time RT-PCR normalization was performed on

the basis of a single gene, beta-actin in our case, to normalize the general expression levels. The normalization according to a single reference is a substantial problem in normalizing expression results. But a normalization according to more references is time consuming, expensive and will result in contradictive and confusing results for each housekeeping gene used. In future a model must be developed, which is able to recognize more than one reference in calculation of relative expression levels to overcome this problem and results in a more realistic and reliable normalization in relative quantification. In conclusion, the normalization in array experiments as well as in kinetic RT-PCR is a general problem and needs high input for further improvements of new concepts, to gain more comparability and reliability in gene expression analysis.

Genes identified by a single microarray experiment with a more than 2-fold expression difference cannot be generally accepted as true without a repetition of the experiment or by validation (e.g., by real-time RT-PCR) and genes identified with differences less than 2-fold should not be eliminated as false positive without repetition or powerful validation. Real-time RT-PCR is well suited to validate and confirm microarray results, because it is rapid, fully quantitative, and requires less than 1000-fold RNA than the microarray experiment [31,32,42]. For verification a relative quantification strategy was applied [36,43], which is based on the expression levels of a target gene vs. a reference gene (housekeeping gene) and adequate for investigation of physiological changes in gene expression levels. A great simplification for the determination at the mRNA levels of the parameters was achieved by using relative quantification and a simple mathematical model [37,41,43]. In the applied mathematical model (equation 4) the mean target gene expression is normalized by a non regulated mean beta-actin gene expression [39]. Real-time RT-PCR in combination with REST is the method of choice for any experiments requiring sensitive, specific and reproducible quantification of mRNA. The software developed, based on the described mathematical model, exhibits suitable reliability as well as reproducibility in individual runs, confirmed by high accuracy and low variation independent of huge template concentration variations [39]. Housekeeping genes are present in all nucleated cell types since they are necessary for basic cell survival. Mostly mRNA synthesis of these genes is considered to be stable in various tissues [44,45,46]. Herein the stable beta-actin expression was chosen as a reference gene to normalize the real-time RT-PCR data. A constant beta-actin expression under Zn deficiency could be confirmed by earlier array experiments [15] and competitive RT-PCR [20]. However each available non regulated reference gene can be used for normalization.

For the determination of the CP the *"second derivate maximum method"* was performed [38], where CP will be measured at the maximum increase or acceleration of fluorescence, even if the fluorescence levels between curves are different [47]. A linear relationship between the CP and the log of the start molecules input in the kinetic RT-PCR reaction is given [48,49]. Therefore quantification will always occur during exponential phase and it will be not affected by any reaction components becoming limited in the plateau phase [40].

5.2. Physiological considerations

The physiological status of the animals was characterized by the absence of growth and a constant feed intake matched to the maintenance requirement of energy [21]. As reported elsewhere [50] metabolic markers as well as blood plasma levels of growth related hormones, growth hormone (GH) and insulin like growth factor-1 (IGF-1), including the expression levels of their receptor proteins (GH-receptor and IGF-1-receptor) remained unchanged during the entire experiment. These results are in contrast with earlier findings where IGF-1 declines in cell culture [50,51] as well as young growing rats [52,53]. Consequently, there was no interaction between Zn deficiency *per se* and the metabolism *in vivo*, as it is usually the case in an animal model based on fast growing individuals (e.g., hormonal disorders due to standstill of growth, suppression of feed intake). Nevertheless, Zn deficiency was evident from a negative Zn retention, the quantitative Zn mobilization from storage tissues (mainly skeleton) and severely reduced plasma Zn concentrations and alkaline phosphatase activities especially at the end of the study [21].

MT expression of MT-1 and MT-2 was measured in microarray and kinetic RT-PCR experiments. As observed in other studies [8,9,20,53–55] and array experiments [15,16] the MT expression was severely down-regulated in liver [53] as well as jejunum [54]. This leads to the conclusion, that MT might be a sensitive marker on the expression level for Zn deficiency [55]. The expression and localization of MT in small intestine indicates a function of Zn and MT in gut immunity and intestinal mucosal cell turnover [13,55,56].

Zn performs a number of unique functions in immunology, which distinguishes it from other nutrient trace elements. Zn enhances the humoral and cell mediated immunity by facilitating proliferative reactions induced by different mitogens and Zn dependent transcription factors [55,57–60]. Cell mediated immunity, antibody reaction and antibody affinity, early B cell development, complement system and phagocytose activity are perceptibly diminished under Zn deficiency [59,61]. Herein jejunum was shown to be a very Zn sensitive tissue with regard to the expression results of immunological relevant genes. 12 of the proposed candidate genes, derived from array analysis, are directly involved in the immunological response cascade of the jejunum, either known as cluster of differentiation (CD) or as immunological relevant chemoattractant. In array experiment the surface markers for B cells were all ~3-fold down-regulated (Table 3); namely: B cell antigen (CD 19), complement receptor type 2 (CD 21), B cell receptor CD 22,

B cell differentiation antigen CD 72, leukocyte antigen CD 37, and CD 72. As shown earlier in mice [61,62] and human cell culture [60,64,65] Zn deficiency has a major effect on the early B cell development and leads to a decline of B cells. This leads to the hypothesis that Zn deficiency causes a reduction of B cells and as a consequence to a reduction in intestinal antibody production. Beside this, Zn is altering the immune function and influences the expression of various chemokines as well as interleukins (IL) [63]. The B lymphocyte chemo-attractant (BCA-1) and the chemokine receptor type 4 (CXC 4) were down-regulated in jejunum (−2.27 and −2.44-fold), at least in the array experiments. Some of the IL, e.g., IL-2 and their receptors are under the control of the Zn dependent transcription factor NF-κB [60,65]. Herein the IL-2 receptor in the absence of Zn is rather repressed (−2.13-fold) in the array.

In comparison to previously published array experiments in small intestine and thymus of growing Zn deficiency rats and mice, only two of the candidate genes found herein match with previously mentioned candidate genes [14–16,53]. This might be due to the different gene configuration of the used arrays as well as on the performed experiment itself. In this study glutathione S-transferase (GST) subtypes (class-a Π, τ) were shown to be down-regulated in liver (−1.64 to 1.96-fold) and in jejunum (−1.28 to −1.11-fold). This could be confirmed by previous publication [15,53] where the subunits GST 8 (class-alpha), GST Yb and microsomal subunit (GST 12) were suppressed under Zn deficiency (−1.5 to −1.7-fold). The second candidate gene found in earlier publication is protein kinase C (PKC). Activation of PKC could be demonstrated to be Zn mediated. PKC itself phosphorylates a variety of target proteins which control cell growth and differentiation. Is has been demonstrated, that Zn activates PKC and contributes to its binding to plasma membranes in T lymphocytes and therefore control the activity of T lymphocytes. Therefore the observed down-regulation of protein kinase C expression (−2.22-fold) under Zn deficiency is in good agreement with earlier published data [66,67]. The last candidate gene found in previous publication was the Zn transporter 2 (ZnT-2) which was 1.9-fold down-regulated [15]. These findings could be verified by own previous studies of various Zn transporters (ZnT-1 to ZnT-4), where ZnT-2 mRNA was down-regulated in the following tissues over the 29 day Zn depletion: 5.5-fold in jejunum, 1.7-fold in liver, and 3.0-fold in muscularity [6].

6. Conclusion

Our data demonstrate that the combination of microarray and real time RT-PCR experiments represents a powerful approach, that summarizes the advantages of both quantification systems - high throughput of the microarray and sensitivity of the real-time RT-PCR. The results demonstrate the feasibility and utility of both methodologies to genome wide exploration of gene expression patterns. But, normalization in array experiments as well as in kinetic RT-PCR is a general problem and needs high input of further improvements of new concepts, to gain more comparability and reliability in gene expression analysis.

The expression results indicate the existence of individual expression pattern in liver and jejunum and their tissue specific regulation under Zn deficiency. Jejunum represents a very Zn sensitive tissue with regard to the expression results of immunological relevant genes. Our results imply that some genes are expressed constitutively, whereas others are highly regulated in tissues responsible for Zn homeostasis. Finally, MT subtype 1 and 2 represent potent candidate genes as markers for Zn deficiency.

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References

[1] Gordon EF, Gordon RC, Passal DB. Zinc metabolism: basic, clinical, and behavioral aspects. J Pediatr 1981;99:341–9.
[2] Swenerton H, Hurley LS. Severe zinc deficiency in male and female rats. J Nutr 1968;95:8–18.
[3] Prasad AS. Zinc: the biology and therapeutics of an ion. Ann Intern Med 1996;125(2):142–4.
[4] Reyes JG. Zinc transport in mammalian cells. Am J Physiol 1996; 270:C401–10.
[5] Liuzzi JP, Blanchard RK, Cousins RJ. Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats. J Nutr 2001;131:46–52.
[6] Pfaffl, MW, Windisch, W. Influence of zinc deficiency on the mRNA expression of zinc transporters in adult rats. Trace Elem. Med Biol. 2003;18(1) (in press).
[7] Brady FO. The physiological function of Metallothionein. Trends Biochem Sci 1982;7:143–5.
[8] Sato M, Mehra RK, Bremner I. Measurement of plasma metallothionein-I in the assessment of the zinc status of zinc-deficient and stressed rats. J Nutr 1984;114(9):1683–9.
[9] Bremner I. Nutritional and physiological significance of metallothionein. Experientia Suppl 1987;52:81–107.
[10] Kirchgessner M. Underwood Memorial Lecture: Homeostasis and Homeorhesis in Trace Element Metabolism. In: Anke M, Meissner D, Mills C F, editors. Trace Elements in Man and Animals TEMA 8. Gersdorf, Germany: Verlag Media Touristik, 1993. pp. 4–21.
[11] Windisch W, Kirchgessner M. Anpassung des Zinkstoffwechsels und des Zn-Austauschs im Ganzkörper 65Zn-markierter Ratten an eine variierende Zinkaufnahme. J Anim Physiol a Anim Nutr 1995;74: 101–12.
[12] Windisch W, Kirchgessner M. Zinkverteilung und Zinkaustausch im Gewebe 65Zn-markierter Ratten. J Anim Physiol a Anim Nutr 1995; 74:113–22.
[13] Bertholf RL. Zinc. In: Seiler HG, Sigel H, editors. Handbook of Toxicity of inorganic compounds. New York: Marcel Dekker Inc, 2000. pp. 788–800.

[14] Blanchard RK, Cousins RJ. Differential display of intestinal mRNAs regulated by dietary zinc. Proc Natl Acad Sci U S A 1996;93(14): 6863–8.
[15] Blanchard RK, Moore JB, Green CL, Cousins RJ. Modulation of intestinal gene expression by dietary zinc status: effectiveness of cDNA arrays for expression profiling of a single nutrient deficiency. Proc Natl Acad Sci U S A. 2001;98(24):13507–13.
[16] Moore JB, Blanchard RK, McCormack WT, Cousins RJ. cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. J Nutr 2001;131(12):3189–96.
[17] Vignolini F, Nobili F, Mengheri E. Involvement of interleukin-1beta in zinc deficiency-induced intestinal damage and beneficial effect of cyclosporine A. Life Sci 1998;62(2):131–41.
[18] Clifford KS, MacDonald MJ. Survey of mRNAs encoding zinc transporters and other metal complexing proteins in pancreatic islets of rats from birth to adulthood: similar patterns in the Sprague-Dawley and Wistar BB strains. Diabetes Res Clin Pract 2000;49(2-3):77–85.
[19] Xu Z, Kawai M, Bandiera SM, Chang TK. Influence of dietary zinc deficiency during development on hepatic CYP2C11, CYP2C12, CYP3A2, CYP3A9, and CYP3A18 expression in postpubertal male rats. Biochem Pharmacol 2001;62(9):1283–91.
[20] Allan AK, Hawksworth GM, Woodhouse LR, Sutherland B, King JC, Beattie JH. Lymphocyte metallothionein mRNA responds to marginal zinc intake in human volunteers. Br J Nutr 2000;84(5):747–56.
[21] Windisch, W. Time course of changes in Zinc metabolism induced by Zinc deficiency in ^{65}Zinc labelled, non-growing rats as a model to adult individuals. Trace Elem Med Biol 2003;18(1) (in press).
[22] Windisch W, Kirchgessner M. Tissue Zn distribution and Zn exchange in adult rats at Zn deficiency induced by dietary phytate additions. J Anim Physiol a Anim Nutr 1999;82:116–24.
[23] Windisch W, Kirchgessner M. Zn absorption and excretion in adult rats at Zn deficiency induced by dietary phytate additions. J Anim Physiol a Anim Nutr 1999;82:106–15.
[24] Windisch W. Homeostatic reactions of quantitative Zn metabolism on deficiency and subsequent repletion with Zn in 65Zn labeled adult rats. Trace Elements and Electrolytes 2001;18:128–33.
[25] Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995;270(5235):368–71.
[26] DeRisi JL, Iyer VR. Genomics and array technology. Curr Opin Oncol 1999;11(1):76–9.
[27] DeRisi JL, Iyer VR, Brown PO. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 1997;278(5338):680–6.
[28] Harrington CA, Rosenow C, Retief J. Monitoring gene expression using DNA microarrays. Curr Opin Microbiol 2000;3(3):285–91.
[29] Bosio A, Knorr C, Janssen U, Gebel S, Haussmann HJ, Muller T. Kinetics of gene expression profiling in Swiss 3T3 cells exposed to aqueous extracts of cigarette smoke. Carcinogenesis 2002;23(5):741–748.
[30] Pfaffl MW, Hageleit M. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol Lett 2001;23:275–82.
[31] Rajeevan MS, Ranamukhaarachchi DG, Vernon SD, Unger ER. Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. Methods 2001;25(4):443–51.
[32] Rajeevan MS, Vernon SD, Taysavang N, Unger ER. Validation of array-based gene expression profiles by real-time (kinetic) RT-PCR. J Mol Diagn 2001;3(1):31–6.
[33] Bosio, A, Stoffel, W, Stoffel, M. Device for the parallel identification and quantification of polynucleic acids. In EP0965647: Memorec Stoffel GmbH, 1999.
[34] Tomiuk S, Hofmann K. Microarray probe selection strategies. Brief Bioinform 2001;2(4):329–40.
[35] Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 2001;29(4):365–71.
[36] Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 (delta delta CT – Method). Methods 2001;25(4):402–8.
[37] ABI Prism 7700 Sequence detection System User Bulletin #2 (2001) Relative quantification of gene expression. http://docs.appliedbiosystems.com/ pebiodocs/04303859.pdf.
[38] LightCycler Software, Version 3.5 (2001) Roche Molecular Biochemicals.
[39] Pfaffl MW, Horgan GW, Dempfle L. Relative Expression Software Tool (REST©) for group wise comparison and statistical analysis of relative expression results in real-time PCR30. Nucleic Acids Research 2002(9),e36–45.
[40] Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25:169–93.
[41] Schmittgen TD. Real-time quantitative PCR. Methods 2001;25(4): 383–5.
[42] Al-Taher A, Bashein A, Nolan T, Hollingsworth M, Brady G. Global cDNA amplification combined with real-time RT-PCR: accurate quantification of multiple human potassium channel genes at the single cell level. Yeast 2000;17(3):201–10.
[43] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(9):2002–7.
[44] Marten NW, Burke EJ, Hayden JM, Straus DS. Effect of amino acid limitation on the expression of 19 genes in rat hepatoma cells. FASEB J 1994;8:538–44.
[45] Foss DL, Baarsch MJ, Murtaugh MP. Regulation of hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase and beta-actin mRNA expression in porcine immune cells and tissues. Anim Biotechnol 1998;9:67–78.
[46] Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E Housekeeping genes as internal standards: use and limits. J Biotechnol 1999;75:291–5.
[47] Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 1998;11:1026–30.
[48] Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res 1996;6:1095–101.
[49] Rasmussen R. Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K, editors. Rapid Cycle Real-time PCR, Methods and Applications. Springer Press, Heidelberg; ISBN 3-540-66736-9, 2001; pp. 21–34.
[50] Pfaffl MW, Bruckmaier R, Windisch W. Metabolic effects of zinc deficiency on the somatotropic axis in non-growing rats as a new animal model to adult individuals. J Animal Sci 2002a;80, supp 1p. 351.
[51] Lefebvre D, Beckers F, Ketelslegers JM, Thissen JP. Zinc regulation of insulin-like growth factor-I (IGF-I), growth hormone receptor (GHR) and binding protein (GHBP) gene expression in rat cultured hepatocytes. Mol Cell Endocrinol 1998;138(1-2):127–36.
[52] Dorup I, Flyvbjerg A, Everts ME, Clausen T. Role of insulin-like growth factor-1 and growth hormone in growth inhibition induced by magnesium and zinc deficiencies. Br J Nutr 1991;66(3):505–21.
[53] tom Dieck H, Doring F, Roth HP, Daniel H. Changes in rat hepatic gene expression in response to zinc deficiency as assessed by DNA arrays. J Nutr 2003;133(4):1004–10.
[54] Andrews GK. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem Pharmacol 2000;59(1):95–104.
[55] Cousins RJ, Blanchard RK, Moore JB, Cui L, Green CL, Liuzzi JP,

Cao J, Bobo JA. Regulation of zinc metabolism and genomic outcomes. J Nutr 2003;133(5):1521S–6S.
[56] Szczurek EI, Bjornsson CS, Taylor CG. Dietary zinc deficiency and repletion modulate metallothionein immunolocalization and concentration in small intestine and liver of rats. J Nutr 2001;131(8):2132–8.
[57] Kruse-Jarres JD. The significance of zinc for humoral and cellular immunity. J Trace Elem Electrolytes Health Dis 1989;3(1):1–8.
[58] Fraker PJ, King LE, Laakko T, Vollmer TL. The dynamic link between the integrity of the immune system and zinc status. J Nutr 2000;130(5S Suppl),1399S–406S.
[59] Kirstetter P, Thomas M, Dierich A, Kastner P, Chan S. Ikaros is critical for B cell differentiation and function. Eur J Immunol 2002; 32(3):720–30.
[60] Prasad AS, Bao B, Beck FW, Sarkar FH. Zinc activates NF-kappaB in HUT-78 cells. J Lab Clin Med 2001;138(4):250–6.
[61] King LE, Osati-Ashtiani F, Fraker PJ. Depletion of cells of the B lineage in the bone marrow of zinc-deficient mice. Immunology 1995;85(1):69–73.
[62] Osati-Ashtiani F, King LE, Fraker PJ. Variance in the resistance of murine early bone marrow B cells to a deficiency in zinc. Immunology 1989;94(1):94–100.
[63] Rink L, Kirchner H. Zinc-altered immune function and cytokine production. J Nutr 2000;130(5S Suppl):1407S–11S.
[64] Prasad AS. Effects of zinc deficiency on Th1 and Th2 cytokine shifts182. J Infect Dis 2000; 182 Suppl 1, S62–8.
[65] Prasad AS, Bao B, Beck FW, Sarkar FH. Zinc enhances the expression of interleukin-2 and interleukin-2 receptors in HUT-78 cells by way of NF-kappaB activation. J Lab Clin Med 2002; 140(4):272–89.
[66] Csermely P, Szamel M, Resch K, Somogyi J. Zinc can increase the activity of protein kinase C and contributes to its binding to plasma membranes in T lymphocytes. J Biol Chem 1988;263(14): 6487–90.
[67] Zalewski PD, Forbes IJ, Giannakis C, Cowled PA, Betts WH. Synergy between zinc and phorbol ester in translocation of protein kinase C to cytoskeleton. FEBS Lett 1990;273(1-2):131–4.