Identification of Genes Responsive to Intracellular Zinc Depletion in the Human Colon Adenocarcinoma Cell Line HT-29[1]

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ABSTRACT Zinc is essential for the structural and functional integrity of cells and plays a pivotal role in the control of gene expression. To identify genes with altered mRNA expression level after zinc depletion, we employed oligonucleotide arrays with ~10,000 targets and used the human colon adenocarcinoma epithelial cell line HT-29 as a model. A low intracellular zinc concentration caused alterations in the steady-state mRNA levels of 309 genes at a threshold factor of 2.0. Northern blot analysis and/or real-time RT-PCR confirmed the array results for 12 of 14 selected targets. Genes identified as regulated based on microarray data encode mainly proteins involved in central pathways of intermediary metabolism (79 genes) including protein metabolism (21). We also identified five groups of genes important for basic cellular functions such as signaling (30), cell cycle control and growth (15), vesicular trafficking (15), cell-cell interaction (13), cytoskeleton (10) and transcription control (19). The latter group comprises several zinc finger–containing transcription factors of which the Kruppel-like factor 4 showed the most pronounced changes. Western blot analysis confirmed the increased expression level of this protein in cells grown under low zinc conditions. Our findings in a homogenous cell population demonstrate that the molecular mechanisms by which cellular functions are altered at a low zinc status, occur via pleiotropic effects on gene expression. In conclusion, the pattern of zinc-affected genes may represent a reference for further studies to define the zinc regulon in mammalian cells. J. Nutr. 134: 57–62, 2004.

KEY WORDS: • *zinc depletion* • *gene expression* • *HT-29 cells* • *DNA array*

Zinc is an essential trace element with cofactor functions in a large number of proteins of intermediary metabolism, hormone secretion pathways and immune defense mechanisms (1). As a cofactor of transcription factors, it is also involved in the control of gene expression (2). On the basis of its multiple biological functions, zinc deficiency causes a wide variety of symptoms including retarded growth, diarrhea, anorexia, impaired immunity, skin lesions and abnormal development (3). Despite decades of research, the molecular targets leading to the pleiotropic effects of zinc deficiency have been identified only partially. Within the last few years, subtracted library hybridization (4) and mRNA differential display (5) approaches have led to the identification of zinc-sensitive genes in animal models of dietary zinc deficiency. More recently, DNA array analysis identified mammalian genes in small intestine, thymus and hepatocytes that respond with altered expression level to changes in zinc status (6–8). Some of the identified genes encode proteins involved in intestinal fluid secretion, signal transduction pathways that control immune response, growth and energy metabolism, and it was suggested that their regulation may contribute to the development of zinc deficiency symptoms in mammals.

Much of our basic understanding regarding zinc-regulated gene expression comes from studies in bacteria and yeast. In *Escherichia coli* (9,10), *Bacillus subtilis* (11,12) and *Saccharomyces cerevisiae* (13), several metalloregulatory proteins acting as zinc sensors, and transcriptional activators/repressors were identified as key components of zinc-dependent gene expression. These zinc finger–containing transcription factors regulate not only zinc-sensitive genes, but also control their own transcription through a positive autoregulatory mechanism. In mammalian cells, the metal-responsive transcription factor 1 (MTF-1)[3] (14) was recognized as a zinc-sensory transcriptional activator. MTF-1 binds to zinc-sensing gene promoter elements, also known as metal-responsive elements (MRE), and induces gene transcription of key target genes such as metallothionein-I and -II (MT-I, -II). Interestingly, disruption of the MTF-1 gene in mice is lethal, whereas animals lacking both, MT-I and -II, are viable (15–18). This suggests that MTF-1 controls expression of important genes other than MT-I and -II. In addition to the regulation of gene transcription via MTF-1, other zinc-sensing metalloregulatory proteins may exist. Very recently, MTF-2, an ortholog of MTF-1, was identified as a zinc-regulated gene (19). Many zinc-regulated

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[3] Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KLF4, Kruppel-like factor 4; MRE, metal-responsive element; MT, metallothionein; MTF, metal-responsive transcription factor; PLAB, prostate differentiation factor; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine.

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genes, however, could be influenced by alterations of signal transduction pathways or by other indirect mechanisms (20). To identify zinc-sensitive genes in mammalian cells we used zinc-depleted HT-29 cells, a human colon adenocarcinoma cell line, and employed oligonucleotide arrays containing ~10,000 genes. In comparison to complex tissues obtained from zinc-deficient animals, our approach uses a homogenous cell population that can be used under standardized conditions to define zinc-dependent gene expression control on a cellular level.

MATERIALS AND METHODS

Cell culture. HT-29 cells were provided by American Type Culture Collection (ATCC, Rockville, MD) and were used between passages 150 and 200. Cells were cultured as described by Wenzel et al. (21). The zinc concentration in the medium after addition of the serum was ~3.7 μmol/L as determined by atomic absorption spectrometry. To reduce the intracellular zinc concentration, 4 μmol/L of the membrane-permeable Zn^{2+}-chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN; Sigma-Aldrich Chemie, Steinheim, Germany) was added to the culture medium; in control experiments, 4 μmol/L zinc (as $ZnCl_2$) was re-added to TPEN-treated medium for adjusting Zn concentration to normal growth conditions.

Determination of intracellular zinc concentration. Total cellular zinc contents were determined by an atomic absorption spectrophotometer (model 5100, Perkin Elmer, Überlingen, Germany). Briefly, cells were grown for 16 h under conditions of low and normal zinc. Cells were harvested, and 20 μL was set aside for the determination of total cell numbers using a Neubauer chamber. Cells were washed with PBS and 10 mmol/L EDTA (in PBS) to remove loosely bound zinc. Cells were lysed by 6% Triton X-100 in isotonic NaCl, and cellular zinc content was measured by air acetylene flame atomic absorption spectrophotometry. Intracellular free zinc concentrations were measured using the zinc-sensitive dye Newport Green diacetate (Bioprobes, Leiden, Netherlands) according to the method described by Sensi et al. with slight modifications (22). Briefly, cells were grown under low and normal zinc conditions as described above. Cells were loaded in the dark with 5 μmol/L Newport Green diacetate in HSS (HSS, composition in mmol/L: 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 20 Hepes, 15 glucose, 1.8 $CaCl_2$, 10 NaOH, pH 7.4) for 30 min at 37°C. Cells were then washed with HSS, kept in the dark for an additional 30 min at 37°C, and fluorescence emission intensity due to the binding of intracellular free zinc was measured at 530 nm after excitation at 485 nm using a fluorescence multiwell-plate reader (Fluoroskan Ascent, Labsystems, Bornheim-Hersel, Germany).

Proliferation and cell integrity. Methods to assess proliferation and cell integrity were described previously (21). Briefly, HT-29 cells were grown under normal and low zinc conditions. Cell counting for determination of proliferation and cell integrity assessment was based on SYTOX-Green (Bioprobes), which becomes fluorescent after DNA binding. Therefore, cells were incubated with SYTOX-Green to determine the number of cells with impaired integrity based on a calibration curve. Cells were then lysed by 6% Triton X-100 in isotonic NaCl and total cell numbers were determined. The percentage of cells with impaired integrity (based on permeability for SYTOX-Green) in a cell population was determined in relation to the fluorescence measured after the solubilization of cells that assessed the total cell count.

Detection of apoptosis. Membrane permeability as an early apoptosis marker and nuclear fragmentation as a late apoptosis marker were assessed as described elsewhere (23). Apoptosis was determined in HT-29 cells after incubation under low and normal zinc conditions for 16 h.

MWG Pan Human 10K array. Oligonucleotide arrays on glass slides containing 9850 gene-specific oligonucleotide probes (50 mer) were obtained from MWG Biotech AG (Ebersberg, Germany). RNA preparation, reverse transcription, labeling and hybridization was performed according to the recommendations of the manufacturer. Total RNA from either control or zinc-deprived cells from three independent experiments was pooled, and reverse transcription in the presence of either Cy3- or Cy5-labeled dCTP (Amersham Bioscience Europe, Freiburg, Germany) was performed to produce fluorescence labeled first-strand cDNAs. Arrays were scanned (Affymetrix 428 Array Scanner, Santa Clara, CA) under dried conditions. The data obtained were normalized and analyzed using ImaGene 4.2 software (BioDiscovery, Los Angeles, CA). Three independent hybridizations were carried out. Genes were considered as up- or downregulated if the change was twofold or greater in at least two hybridizations. In most cases Northern blot analysis as well as real-time RT-PCR showed similar or even greater changes in expression levels than those observed on microarrays.

Northern blot analysis. Northern blot analysis was carried out according to an established capillary blotting method (24). Total RNA (5 μg/lane) was size-fractionated on a denaturating formaldehyde gel and transferred onto a Hybond-N+ nylon membrane (Amersham Bioscience Europe). The cDNA fragments representing unique open reading frames of the following genes were used for hybridization: Kruppel-like factor 4 (KLF4) (accession no.: NM_004235, nucleotides 590–968); prostate differentiation factor (NM_004864, 161–550); NADH dehydrogenase Fe-S protein 4 (NM_002495, 121–317); zinc finger protein 165 (NM_003447, 398–762); MT-I (K01383, 124–366); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (BL029618; 549-1001). The cDNA fragments were randomly labeled with [α^{32}P]-dATP. Hybridization and further blot processing were carried out as described elsewhere (8). Blots were reprobed for GAPDH and the resulting signal was used for normalization.

LightCycler real-time RT-PCR. Quantitative RT-PCR was performed as described earlier (25). Briefly, 1 μg of total RNA from HT-29 cells, grown under normal and low zinc conditions, was reverse transcribed to generate cDNA pools, and 3.3 ng reverse-transcribed total RNA was added to each PCR reaction. The relative amount of target mRNA normalized to 18S rRNA was calculated according to the method described by Pfaffl et al. (26). Primer design was done using the HUSAR software at DKFZ. Blast search in the published sequence database GenBank (27) revealed that primers are gene specific; if possible, primers that spanned at least one intron were chosen. Based on these criteria, the following primers were used: Kruppel-like factor 4 (forward primer 590–609 (position in the open reading frame); reverse primer 728–711); zink finger protein 165 (398–419,762–744); H4 histone family member (45–63,301–284); serin protease inhibitor (16–37,214–194); proteasome subunit (296–315,426–406); cathepsin H (415–434,580–560); hspc163 protein (182–203,335–315); aspartate aminotransferase 2 (558–577,756–739); NADH dehydrogenase Fe-S protein 4 (121–140,317–298); sialidase 1 (547–564,720–703); DEAD/H box polypeptide 1 (1087–1108,1378–1359); and MT-I (81–100,186–166), 18S (1146–1165,1350–1331; position in the 18S rRNA).

Western blot analysis. Immunoblotting was carried out using an established standard blotting method (24). Protein preparations (60 μg protein/lane) were separated by 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking in TBS/0.1% Tween-20 containing 3% nonfat dry milk, the blot was immunostained with anti-KLF4 antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), a horseradish peroxidase–conjugated anti-goat IgG antibody (1:1000 dilution; Santa Cruz) and 3-amino-9-ethylcarbazole. Protein concentration was determined by optical density at 600 nm using the Bio-Rad protein assay (Bio-Rad, München, Germany). Equal protein loading and transfer to the membrane were assessed by ponceau S-staining of proteins before further blot processing.

Statistical analysis. Calculations were done using the software Prism 2.01 (GraphPad Software, Los Angeles, CA). Results for cellular zinc concentration, proliferation and cell integrity were analyzed using unpaired Student's *t* test and were considered significantly different at P-value < 0.05. Data are means ± SD.

RESULTS

Effects of the Zn^{2+}-chelator TPEN on cellular zinc concentration, MT-I expression, cell proliferation and cell integrity. To deplete cells of intracellular zinc, HT-29 cells were exposed for 16 h to 4 μmol/L of the membrane-perme-

TABLE 1

Effects of zinc deprivation on total and intracellular free zinc levels and proliferation in human HT-29 cells[1]

	+Zn[2]	−Zn[3]
Total cellular zinc, *pmol/10⁶ cells*	348.3 ± 21.9	125.2 ± 31.8*
Intracellular free zinc,[4] *FU/10⁶ cells*	4.5 ± 0.1	2.7 ± 0.1**
Total cell number	145,695 ± 7912	107,661 ± 8016**

[1] Values are means ± SD, $n = 3–6$. * Different from +Zn, $P < 0.05$; ** different from +Zn, $P < 0.001$.
[2] +Zn, zinc-adequate.
[3] −Zn, zinc-depleted.
[4] FU, fluorescence units.

able Zn^{2+}-chelator TPEN. Under those conditions, the chelation of ions other than zinc is very low (28,29). In control experiments, TPEN was added together with 4 μmol/L zinc. The resulting cellular (extra- and intracellular) zinc concentration was significantly decreased in TPEN-treated cells (**Table 1**). When the treated cells were replenished with zinc, the intracellular zinc concentration increased to the same level as that in cells cultured with normal medium (normal medium: 4.6 fluorescence units, medium replenished with zinc: 4.5 fluorescence units). As observed previously in other cells (30), zinc depletion also significantly decreased cell proliferation in HT-29 cells (Table 1). However, cell integrity (Table 1) as well as early and late apoptosis events (**Fig. 1**) were not affected by low intracellular zinc concentrations. Moreover, the expression level of the MT-I, considered to be a sensitive indicator of intracellular zinc status (31,32), was drastically reduced in zinc-depleted cells (**Fig. 2**). Therefore, the cell model used to simulate a zinc-deficient state appears suitable and does not impair cell integrity and apoptosis.

Identification of genes responsive to a low intracellular zinc concentration. For identifying genes responsive to a low intracellular zinc concentration, we carried out oligonucleotide array analysis of the transcriptome of HT-29 cells grown for 16 h under low or normal zinc conditions. Of the ~10,000 genes analyzed, changes (>twofold) in the mRNA-levels were detected for 309 genes; 231 genes were downregulated, whereas 78 genes were upregulated upon intracellular zinc depletion.[4] The genes can be classified into 8 groups based on the function of the gene products or by homology with previously described proteins in public databases (**Fig. 3**). Identified groups encode for proteins involved in metabolism ($n = 58$), protein metabolism ($n = 21$), transcription ($n = 19$), signaling ($n = 30$), cell growth/cycle ($n = 15$), vesicular trafficking ($n = 15$), cell-cell interaction ($n = 13$) and cytoskeletal homeostasis ($n = 10$). These groups cover 59% of the identified genes; 19% of the identified genes encode for proteins with miscellaneous functions and could not be clustered ($n = 59$). Zinc-dependent regulation was also found for a number of genes that encode for yet hypothetical proteins ($n = 40$) or proteins with unknown function ($n = 29$).

Verification of changes in gene expression. To evaluate the reliability of the array results, we performed Northern blot analysis and real-time RT-PCR for 14 selected genes from different clusters. Alterations in mRNA levels were confirmed for 12 genes in the same direction by quantitative RT-PCR and/or Northern blotting. However, changes in transcript levels were often two- to fourfold higher than those determined on oligonucleotide arrays. In the case of three genes, the zinc finger protein 217 (ZNF217), the prostate differentiation factor (PLAB) and cytochrome P_{450} subfamily VIIa (CYP7A1), a specific PCR product could not be obtained. ZNF217 was not regulated by zinc depletion (**Table 2**) when the mRNA-levels were determined by Northern blot analysis. This, however, is not attributable to the sensitivity in Northern blot analysis

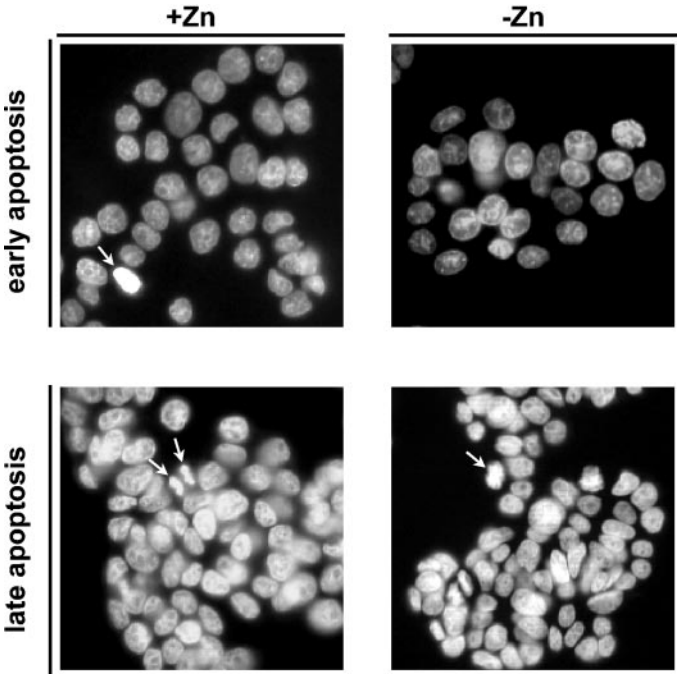

FIGURE 1 Determination of early and late apoptosis events in human HT-29 cells grown for 16 h under normal (+Zn) and low (−Zn) zinc conditions. Arrows indicate apoptotic cells due to membrane disintegration (early apoptosis) or nuclear fragmentation (late apoptosis).

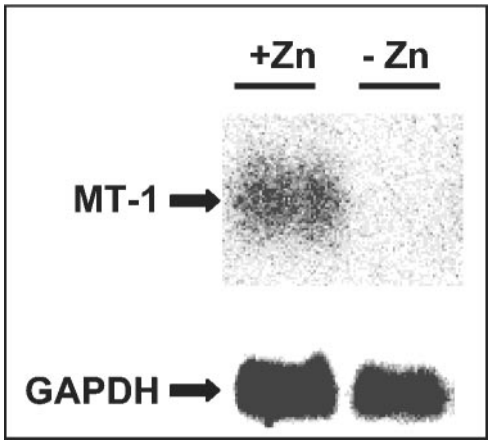

FIGURE 2 Northern blot analysis of metallothionein (MT) mRNA levels in HT-29 cells grown for 16 h under normal (+Zn) and low (−Zn) zinc conditions. Human MT-1 mRNA levels were compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control on the same blot.

[4] A complete list of genes representing different functional classes is available as a supplemental file in the online posting of this paper at www.nutrition.org.

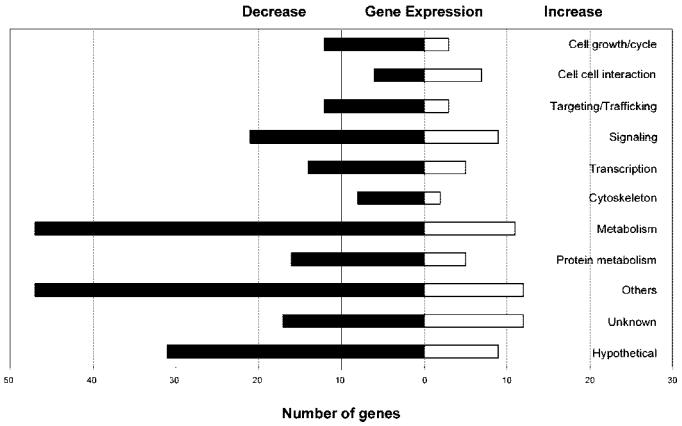

FIGURE 3 Functional classification of genes with altered expression levels in response to low zinc conditions in human HT-29 cells. Open and closed bars represent the number of genes that had increased or decreased mRNA levels in zinc-depleted cells found by array analysis.[4]

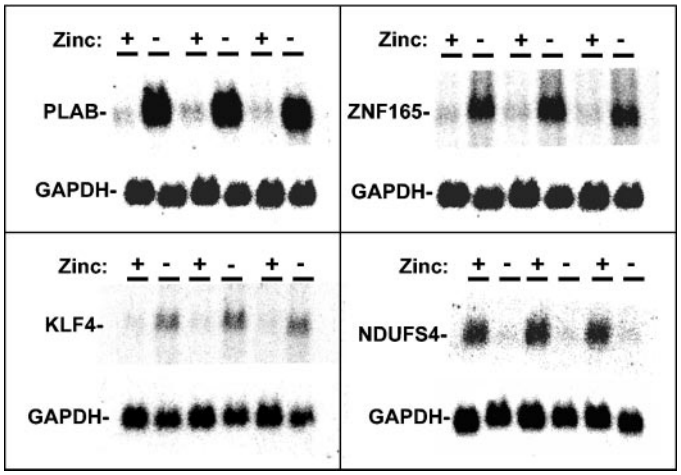

FIGURE 4 Verification of selected genes regulated by low zinc concentration in human HT-29 cells cultured for 16 h under normal (+) or low (−) zinc conditions with Northern blot analysis. The blots were probed for PLAB (prostate differentiation factor), KLF4 (Kruppel-like factor 4), ZNF165 (zinc finger protein 165) and NDUFS4 (NADH dehydrogenase Fe-S protein 4). Each blot was reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as an internal control, $n = 3$.

because those performed for PLAB, zinc finger protein 165 (ZNF165), KLF4 and NADH dehydrogenase Fe-S protein 4 (NDUFS4) confirmed all of the results obtained by oligonucleotide array analysis and RT-PCR (**Fig. 4**).

Regulation of the KLF4 on the protein level. The zinc finger-containing transcription factor KLF4 seems to be an important gene regulated by zinc depletion. Therefore, we carried out Western blot analysis to determine whether the effect observed at the transcript level is reflected in the abundance of KLF4-protein. The KLF4-antibody recognized a single protein band with the appropriate molecular weight in a protein preparation of HT-29 cells that showed a 1.9-fold increased steady-state protein level (**Fig. 5**).

DISCUSSION

In the present study, we used the human colon adenocarcinoma cell line HT-29 to identify zinc-sensitive genes under conditions of a zinc deficiency. The screening based on oligonucleotide arrays showed that the depletion of intracellular zinc altered the mRNA-expression of ~3% of the 9850 genes represented on the array. Changes in expression level of representative transcripts were confirmed independently by

TABLE 2

Changes in mRNA expression levels of selected genes in response to intracellular zinc depletion in human HT-29 cells[1]

Gene[2]	Encoded protein[3]	Function[4]	Fold change at low zinc concentration[5]		
			Array	Northern blot	RT-PCR
NM004235	Kruppel-like factor 4	Transcription	+5.3 ± 0.1	+7.2 ± 0.5	+8.9 ± 1.2
NM003447	Zink finger protein 165	Transcription	+3.2 ± 0.6	+5.2 ± 0.9	+7.3 ± 1.0
NM006526	Zink finger protein 217	Transcription	−4.8 ± 0.2	NR	UP
XM030144*	H4 histone family member	DNA-binding	−8.4 ± 1.5	ND	−4.0 ± 0.1
NM003122	Serin protease inhibitor	Protein degradation	+3.7 ± 0.4	ND	+3.9 ± 0.1
NM002786	Proteasome subunit	Protein degradation	−2.0 ± 0.2	ND	−2.4 ± 0.2
NM004390	Cathepsin H	Protein degradation	−2.3 ± 0.9	ND	−1.4 ± 0.2
NM014184	Hspc163 protein	Protein folding	−4.5 ± 1.6	ND	−19.6 ± 1.2
NM002080	Aspartate aminotransferase 2	Amino acid metabolism	−2.3 ± 1.0	ND	−8.2 ± 1.1
NM000780	Cytochrome P_{450}, subfamily VIIa	Xenobiotic metabolism	−2.5 ± 0.3	ND	UP
NM002495	NADH dehydrogenase Fe-S protein 4	Energy metabolism	−3.3 ± 0.8	−3.2 ± 0.2	−7.1 ± 0.6
NM000434	Sialidase 1	Lipid degradation	+5.0 ± 0.9	ND	+7.0 ± 0.6
NM004939	DEAD/H box polypeptide 1	Apoptosis	−5.5 ± 0.5	ND	−16.2 ± 0.5
NM004864	Prostate differentiation factor	Cell differentiation	+6.9 ± 2.9	+8.6 ± 1.2	UP

[1] List of selected zinc-sensitive genes with increased (+) or decreased (−) expression level. Genes were originally identified by array analysis and were confirmed by Northern blot analysis and/or quantitative RT-PCR.

[2] GenBank accession number. [* This record was removed as a result of standard genome annotation processing. See the genome documentation (46) for further information.]

[3] Name of encoded protein.

[4] Proposed function of the encoded protein.

[5] Magnitude of changes observed by array, Northern blot analysis or real-time RT-PCR. Values are means ± SD, $n = 3$ experiments. ND, not determined, UP, unspecific product obtained, NR, not regulated.

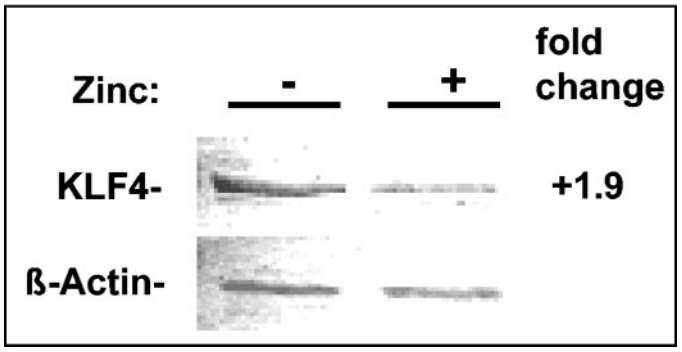

FIGURE 5 Western blot analysis of Kruppel-like factor 4 (KLF4) in human HT-29 cells cultured under low (−) and normal zinc (+) conditions for 16 h. KLF4 protein levels were compared with β-actin as an internal control on the same membrane. Fold change was normalized for β-actin in three independent experiments. A typical blot is shown.

Northern analysis and/or quantitative RT-PCR, demonstrating the reliability of the data derived from the arrays. In most cases, the alternative methods for transcript profiling revealed greater differences than those found by oligonucleotide arrays. Therefore, the defined threshold value of a twofold change in mRNA-expression level appears valid and allows a conservative estimate of the numbers of genes with altered transcript levels upon zinc depletion. Similar changes in global gene expression were found by differential-mRNA display and cDNA array analysis in murine thymocytes and human monocytes (19,33) with ~5% of genes identified as zinc responsive.

Identified genes that responded to zinc status in HT-29 cells could be sorted into eight functional categories. Four of these gene classes (cell growth/cycle, signaling, cytoskeleton, metabolism) were also identified in a recent microarray study examining the effect of zinc deprivation or zinc excess on the expression of 22,216 genes in a human mononuclear cell line (19). Genes within those groups seem to represent a first comprehensive collection of genes that are zinc responsive independent of the cell type. However, which genes among these lists are directly or indirectly regulated by zinc is still not known. Future studies employing bioinformatic approaches such as multiple expectation-maximization for motif elicitation (34) or motif alignment search tool (35) should allow the identification of similar regulatory elements in the promoters of collected genes. Similar strategies were extremely powerful in defining a zinc-responsive regulon in yeast (36). The 46 genes of this regulon are controlled by the metalloregulatory protein Zap1p. In mammalian cells, the zinc finger protein MTF-1 has been identified as a zinc-sensing metalloregulatory protein (18). Zinc binds to MTF-1 and provides a positive signal for the expression of genes that contain metalloregulatory promotor elements. The number of genes regulated by MTF-1 is currently not known, but a first list of genes containing such regulatory elements was recently generated on the basis of computer searches (37). In HT-29 cells, we found 231 genes with lower steady-state expression level upon zinc depletion, and this set of genes may contain numerous targets of MTF-1. MTF-1 is usually upregulated in cellular zinc deficiency as found also in our cell model (data not shown). Recently, the MTF-1 ortholog MTF-2 was shown to be downregulated by zinc depletion in human mononuclear cells (19), and this reciprocal regulation of MTF-1 and MTF-2 was suggested to allow the opposite regulation of gene clusters with enhanced or lowered expression levels. In this sense, MTF-2 could represent a candidate protein that acts at least on some genes of the 78 transcripts found here to be upregulated by zinc depletion. Of course, database searches for metalloregulatory elements and reporter assays of the candidate gene promoters are necessary to define new MTF-1 and MTF-2 target genes.

Of the genes with significantly altered expression level, >20% encode hypothetical proteins or proteins with unknown function. It is interesting to note that among these, seven CGI-genes were regulated by zinc depletion. CGI-genes were identified recently by the comparative gene identification approach (38) and comprise >150 putative full-length gene transcripts. Although the functions of these genes are not yet known, their high degree of homology and conservation from *Caenorhabditis elegans* to humans suggests a fundamental role in the control of cellular processes. The largest gene cluster encompasses 58 genes encoding enzymes of the intermediate metabolism. Remarkably, 81% of these genes were downregulated by zinc depletion, including those of proteins involved in energy metabolism such as ATP synthase (subunit f), cytochrome c, a subcomplex of the NADH dehydrogenase 1 and other dehydrogenases. This observation suggests that the cellular energy metabolism in HT-29 cells is impaired by zinc depletion, and such changes have indeed been demonstrated in different cell types such as hepatocytes (39) and lymphocytes (40). An impaired cellular energy charge may be explained by reduced activity of zinc-dependent enzymes involved in energy-yielding pathways, but may also be mediated at least in part through reduced expression of genes encoding enzymes of energy metabolism. Within the cluster of genes important for metabolism, we identified several zinc-sensitive genes encoding proteins for protein synthesis, protein degradation and amino acid metabolism. Genes such as the translation initiation factor isoforms 4g and 4a (eif4g1, eif4a2), elongin b, proteasome subunit (psmd3), ubiquitin specific protease 7 (usp7) and aspartate aminotransferase 2 (got2) and methionyl-aminopeptidase 2 (metap2) had two- to threefold reduced expression levels in zinc-depleted cells. Consistent with our findings in HT-29, genes involved in protein degradation such as ubiquitin, ubiquitin-conjugating enzyme E2I and the ubiquitin-specific protease 24 were also identified in human mononuclear cells grown under low zinc conditions (19,41). The changes in protein metabolism may reduce protein turnover as observed in different cell types in zinc-deficient animals (42) as well as in the model organism *Candida albicans* (43).

On the basis of experiments in which the zinc requirement for proliferation in cultured cells was examined, Chestes and Boyne (30) hypothesized that zinc was required for proteins important for the cell cycle control. Consistent with this hypothesis, we found 15 zinc-sensitive genes involved in regulating cell growth and cell cycle progression. Although transcript levels of genes that increase proliferation (e.g., cyclin a, cyclin-dependent kinase 8) were downregulated 2.1- to 3.9-fold, genes that suppress cell proliferation such as the cyclin-dependent kinase inhibitor 2d or the growth-arrest and DNA-inducible factor gadd45a were upregulated 2.7- to 4.0-fold in zinc-depleted cells. A variety of studies examining the role of zinc in signaling pathways (20) suggest that zinc depletion could also affect membrane signaling systems and intracellular second messengers that coordinate cell cycle and cell proliferation (44). We also observed that genes encoding proteins for signaling processes (e.g., protein tyrosine phosphatase, phospholipase a2-activating protein) displayed changed expression levels under low zinc conditions. The decreased cell proliferation in zinc-deprived HT-29 cells (Table 1) appears to represent the integrated read-out of these alterations in gene expression of proteins that control the cell cycle. Within that

context, the identified zinc finger–containing transcription factor KLF4 is a particularly interesting gene and could provide a direct link between the cellular zinc status and growth inhibition. KLF4 was cloned as a gene whose expression accompanies a growth arrest (45). KLF4 upregulated expression of several inhibitors of the cell cycle such as cyclin-dependent kinases. It also suppressed the expression of genes that are positive regulators of the cell cycle such as the cyclins. An upregulation of KLF4, therefore, should lead to reduced cell growth. In agreement with this hypothesis, zinc depletion in our cell model caused an increase in the steady-state expression level of the KLF4-mRNA with a simultaneously increased protein level as demonstrated by Western blot analysis (Fig. 5). Altered expression of KLF4 by changes in the cellular zinc status was not observed previously and may provide a new mechanism by which the function of zinc in regulating cell growth is mediated.

In summary, the results of our cell culture study show that an oligonucleotide-based gene expression analysis provides a powerful tool for the identification of genes that respond to a cellular depletion of zinc. The pattern of genes affected by zinc could be a basis for further studies to define the zinc regulon in mammalian cells. In addition, we demonstrated for the first time that expression of KLF-4-mRNA and -protein increases in zinc-depleted cells. This could be a new mechanism to explain the effect of zinc in the regulation of cell growth.

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