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Extremely low copper concentrations affect gene expression profiles of human prostate epithelial cell lines

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ABSTRACT

Although cellular copper metabolism is tightly regulated through a variety of copper transport proteins and chaperones, disturbances in copper homeostasis are involved in several pathological disorders. The aim of this study was to evaluate the effects of extremely low copper concentrations on gene expression profiles of a line of human prostate epithelial cells (RWPE) which grows in the absence of fetal calf serum, a source of variable and unpredictable copper. Cells were exposed to copper(II) sulfate for 24 h at concentrations varying from 10^{-6} to 10^{-17} M and untreated reference cells were exposed to the same volume of copper-free water. Relative gene expression variations between copper-treated and control cells were studied with microarray technology using the Whole Human Genome Array from Agilent. Microarray data demonstrated that copper added to the medium varied gene expression at all concentrations tested. Many genes belonging to functional gene families were modulated by copper, some dose-dependently. Amongst these genes metallothioneins (MT1A and MT2A) were over-expressed at all copper concentrations, MT1M was up-regulated between 10^{-6} and 10^{-9} M, while MT1B, MT1E, MT1G and MT1H were up-regulated between 10^{-6} and 10^{-14} M. The heat shock protein (HSP) gene family showed similar behavior: some HSP genes were constantly up-regulated by copper (HSP90Ad, HSP90B1 and HSPD1) and others only at higher concentrations (HSP90AB1 and HSPA8). Reverse-transcription-PCR analysis, performed on four different genes on five biological replicates for selected genes, on each copper concentration tested, confirmed the trend observed in microarray results. In conclusion, we unexpectedly observed a modulation of gene expression even at extremely diluted copper concentrations, similar to that induced by toxic concentrations, possibly as a result of very tight control of free copper(II) levels inside the cells.

1. Introduction

Transition metal copper (Cu) is an essential trace element for all organisms, given its ability to act as a cofactor for a variety of redox enzymes such as those involved in ATP production, in de-activation of reactive oxygen species and in amine biosynthesis. However, the redox properties of Cu(II) can also cause extensive oxidative damage to lipids, proteins and nucleic acids at high concentrations [1].

In mammals, Cu is an essential element present in the diet, is mainly absorbed in the small intestine through the Cu-transporter-1 protein and is excreted in the bile [2]. Cu absorption, excretion and tissue distribution is regulated by a complex variety of transport proteins and chaperones [3].

The necessity of a tight intracellular Cu control is clearly shown by rare disorders like Menkes and Wilson diseases, caused by Cu deficiency or accumulation, respectively [4]. However, disturbances in Cu metabolism possibly play a role in neurodegenerative disorders such as Alzheimer's and Parkinson's disease [5], osteoporosis and cardiovascular diseases [6] and in cancer, since many types of tumours have Cu concentrations higher than the corresponding normal tissue [7] and ceruloplasmine levels can be correlated with the cancer stage [8,9].

Although there is a lot of information on Cu in biological systems we do not know what are the effects of Cu at low concentrations and the optimal concentration of free Cu within cells is undetermined. It is clear that most organisms have developed mechanisms to avoid Cu overload, including the uptake reduction, enhancement of the export and Cu sequestration in metal-binding proteins such as metallothioneins.In experimental systems *in vitro* the toxic effects of Cu are variable: concentrations of 1–10 μM increase ROS levels in astrocytoma and neuroblastoma cell lines [5], 10 μM induce necrosis in primary hepatocytes [10] and breast cancer cells [11]and

Abbreviations: Cu, copper; HSP, heat shock protein.

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78 μM kill 50% of CHO cells [12]. Even low chronically administered doses (0.21 ppm in water) *in vivo* can induce neurotoxicity in rodents [13].

Cu supplementation, often at low doses, has been proposed for the cure of various ailments, often without a solid scientific basis and Cu overload can result in liver disease, severe neurological problems, renal failure and gastrointestinal bleeding [14], explaining why Cu as a supplement is administered at low doses and with extreme caution.

We wanted to address the problem of the assessment of cellular effects at low concentrations of chemicals. In environmental toxicology we often face the problem of determining possible effects of chemicals which can be a fraction of the lowest estimated toxic doses (LOAEL). A similar problem is encountered when developing therapeutic protocols with active drugs at low doses as a possible alternative to the full dose regimens used for most diseases. In both cases, when the problem shifts from clinical applications to fundamental biological processes, we encounter epistemological and experimental limitations due to the low dimensionality of the substances to be investigated. Therefore, the challenge becomes to study the reactivity of a biological system in limited conditions using the best available methodologies.

Owing to the poor sensitivity of the usual physical and biochemical approaches, we focused our attention on the modulation of gene expression by low concentrations of Cu^{2+} in an immortalized human prostate epithelial cell line (RWPE-1). This choice was determined by the fact that these cells grow in the absence of fetal calf serum, which is an unpredictable source of Cu(II) ions. This model allowed us to study changes in gene expression at concentrations much lower than those associated with classical pharmacological and toxicological responses. The obtained results are reported and discussed in what follows.

2. Material and methods

2.1. Cells

We used a non-neoplastic adult human epithelial prostatic cell line (RWPE-1) obtained from ATCC (Manassa, VA, USA). The cells were originally immortalized with human papillomavirus 18 and grown in keratinocyte-SFM medium (Gibco, Milan, Italy). This type of cell grows easily in the absence of fetal calf serum that contains considerable and unpredictable levels of copper.

Cells were grown in T-75 plastic culture flasks at 37 °C in a 5% CO_2 atmosphere, until 60% confluence was reached and exposed to all experimental Cu concentrations or to the same volume of water for 24 h; at the end of this period, cells were harvested with a trypsin/EDTA solution and stored at −20 °C in RNAlater. Cu treatments were replicated 5 times.

2.2. Preparation of the Cu dilutions

Cu(II) solutions (from 10^{-6} to 10^{-17} M) were prepared by serial dilutions of a 10^{-3} M initial solution of Cu sulfate (Sigma, Milan, Italy) in nuclease-free water (Ambion, Applied Biosystem, Monza, Italy). Reference untreated cells were exposed to the same volume of water used for the preparation of the Cu solutions.

2.3. Analytical procedure

The determination of Cu in the culture medium, in nuclease-free water and in the cellular suspensions was done by plasma mass spectrometry after 1:10 (v/v) dilution of each sample with HNO_3, using the standard addition method and a ICP-AES VARIAN 720-ES spectrometer, employing an ultrasonic CETAC 5000AT+. The detection limit was 0.06 ppb.

2.4. Determination of cytotoxicity

Cytotoxicity was estimated using the neutral red uptake inhibition assay, according to Borenfreund and Puerner [15].

2.5. Microarray analysis

Total RNA was extracted using the RNeasy Mini kit Plus (Qiagen, Milan, Italy). Gene expression analysis was performed using the Agilent Whole Human Genome microarrays, containing 44K spots, corresponding to 41,000 human genes and transcripts. The array permits a complete investigation of human gene expression profiles. About 50 ng of total RNA were labelled (Cu-treated cells with CY5 and control cells with CY3) and hybridized using the Agilent Two-Colour Microarray-Based Gene Expression Analysis protocol. The images were scanned using a Genepix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA); image analysis, spot quality control and normalization were performed using the Feature Extraction software from Agilent Technologies. The Eisen software (freely available at http://rana.stanford.edu) was used to perform a cluster analysis using the K-means method, in order to identify gene clusters representing a possible "Cu signature". All genes found to be differentially expressed ($p < 0.05$), comparing Cu-treated *vs* untreated cells, in at least 80% of the experiments, were included in the analysis. Log-transformed fold changes were arbitrarily clustered into groups of genes having similar expression profiles. The number of nodes was chosen in order to give the largest possible number of similar or different patterns.

To identify biological processes affected by Cu treatment, we used the visualization tool GenMAPP version 2.1 (Gene Map Annotator and Pathway Profiler, http://www.genmapp.org). This is a freely accessible program for viewing and analyzing gene expression data representing biological pathways or other functional gene groups.

2.6. RT-PCR

About 100 ng of total RNA were reverse-transcribed using 100 units of SuperScriptTM II Reverse Transcriptase (Life Technologies, Milan, Italy) and 1× random hexamers (Roche Diagnostics, Monza, Italy). The following genes were tested: HSP90AA1 and HIST1H4B, found to be up-regulated at Cu concentrations from 10^{-6} to 10^{-13} M and down-regulated at lower concentrations (Fig. 2, cluster 3c); HSPA8 and HIST1H1D genes, up-regulated from 10^{-6} to 10^{-9} M and down-regulated at lower concentrations (Fig. 2, cluster 3a). The primers used were: *HSP90AA1 F* 5′-TAT AAG GCA GGC GCG GGG GT-3′, *HSP90AA1 R* 5′-TGC ACC AGC CTG CAA AGC TTC C-3′; *HIST1H4B F* GGC CAT TCG GCG CCT TGC TA-3′, *HIST1H4B R* AAG CCG TAC AGA GTG CGT CCT TGA-3′; *HSPA8 F* TGC TGC TCT TGG ATG TCA CTC CTC-3′, *HSPA8 R* TCC TGG CAT GCC TGT GAG TTC AA-3′; *HIST1H1D F* GCC CAA GAA GGT GGC TGG CG-3′, *HIST1H1D R* CCC GAC TTA GGC TTG GCC GC-3′; *GAPDH F* CCC TCA AGG GCA TCC TGG GCT-3′, *GAPDH R* GCA GGG ACT CCC CAG CAG TGA-3′.

Each of these genes was co-amplified with GAPDH as internal standard. PCRs were carried out using 2 μl of cDNA in a 25 μl total volume containing PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 8 ng/μl of primer, 0.1 ng/μl of GAPDH primers and 1.25 units of Taq polymerase (Sigma–Aldrich, Milan, Italy). The PCR conditions were: 95 °C for 7 min and 35 cycles at 95 °C for 30 s, 60 °C (55 °C for ASP90AA1) for 30 s and 72 °C for 55 s and a final extension at 72 °C for 5 min. The PCR products were separated on a 1.8% agarose gel and visualized by ethidium bromide staining. Gel images were captured by a digital photocamera and the intensity of the bands

analyzed with the Quantity-One software (Bio-Rad, Segrate, Milan, Italy).

3. Results and discussion

The main goal of the present study was to investigate the effects of Cu on gene expression in cells *in vitro* in a large range, including extremely low concentrations. Accordingly, solutions of Cu sulfate (CuII) were added to the culture medium in nuclease-free water to obtain final concentrations ranging from 10^{-6} to 10^{-17} M. In each experiment, control cells from the same batch were treated with the same volume of nuclease-free water.

We determined that the cell culture medium used in our experiments contained $1–2 \times 10^{-10}$ mol/L Cu, using plasma atomic emission spectrometry, whereas the nuclease-free water used for the dilutions had Cu level below the detection limit. Due to the lack of analytical methods with higher sensitivity, it was not possible to determine the exact amount of free Cu present in the incubation media at the low dilutions. Consequently, the experiments were designed in a way that would allow the measurement of biological effects of Cu at concentrations below the chemical detection limit. Therefore, in each experiment, RWPE cells were split into 13 flasks, grown until 60–70% confluence and finally exposed to a fresh medium, supplemented with nuclease-free water or with the same volume of the appropriate Cu sulfate dilutions. At all the concentrations tested Cu did not induce overt cytotoxicity as estimated by the neutral red assay (data not shown).

Initially, we used a single set of biological experiments for microarray analysis. Eventually, to obtain a further estimate of the variability of the system, we performed microarray analyses of five biological replicates of each copper concentration, measuring gene expression in pools of RNAs obtained by mixing equal amounts of RNA from these five replicates. The same replicates were used in RT-PCRs analyses for selected genes.

The analysis performed on a single set of experiments demonstrated that addition of Cu to the medium modulated gene expression at all concentrations, including the most diluted. Fig. 1 shows the percentage of differentially expressed genes ($p < 0.01$) with at least a 2-fold change, comparing Cu-treated *vs* untreated cells, used as a reference in each experiment. The percentage of significantly up- or down-regulated genes was not correlated in a simple fashion with the amount of Cu added to the medium, although higher concentrations of Cu had a tendency to show stronger effects.

The results of clustering microarray data as a function of Cu concentration are shown in Fig. 2. We identified a group of 156 genes (cluster 1) down-regulated (in green) at all Cu concentrations, including a gene codifying for a copper transporter homolog

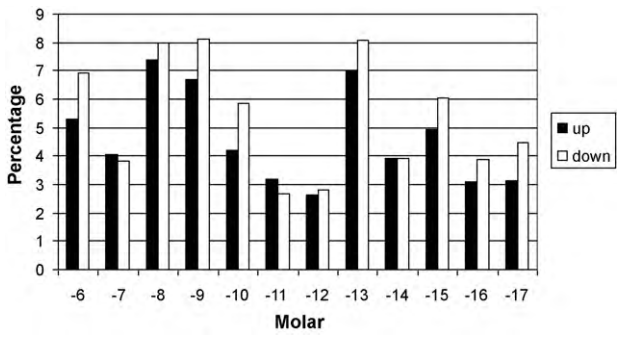

Fig. 1. Percentage of genes significantly modulated by different Cu concentrations with a fold change (FC) of at least 2 ($p < 0.01$) compared to reference cells. The percentage was calculated as a ratio of the total number of up- or down-regulated genes after quality control analysis.

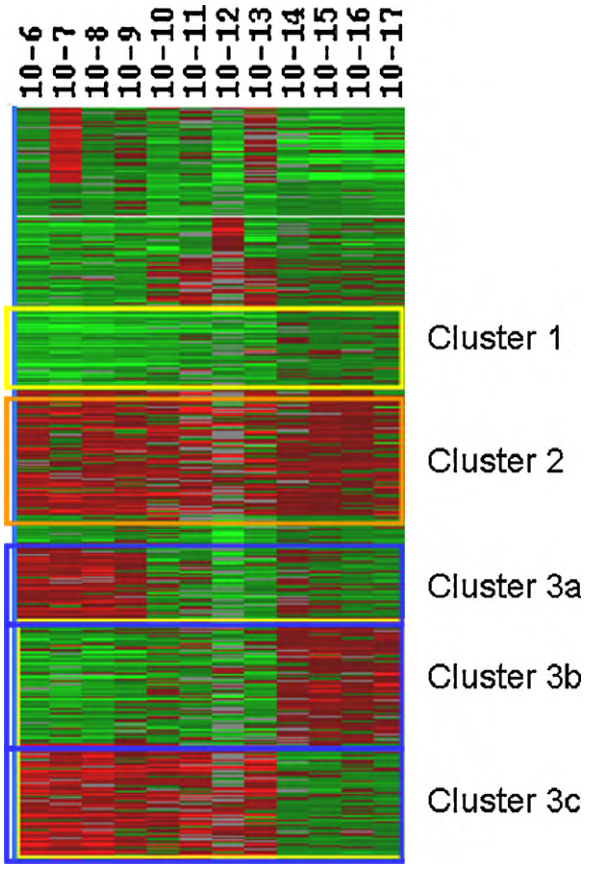

Fig. 2. Clustering of gene expression profiles on a set of cells treated with Cu, compared to untreated reference cells. Microarray data were analyzed by K-means clustering; genes differentially expressed in at least 80% of the experiment, were included in the analysis (horizontal lines); the log2 ratio of gene expression levels (treated/untreated) is color-coded: red indicates up-regulation, green down-regulation, black no change. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in *E. coli* (CUTC) and a metallopeptidase (ADAM17). A second set of 249 genes (cluster 2) was up-regulated (in red) in Cu-treated cells, compared to the controls. In this cluster we found genes codifying for ATPase transporting proteins (ATP6V1H and ATP5E), heat shock proteins (HSP90Ad, HSP90B1 and HSPD1), ubiquitin-conjugating enzymes (UBE2H, UBE2L3, UBLCP1 and UBQLN1), proteasome activators (PSME3), ribosomal proteins (RPL31, RPL34, RPS26, RPS29, RPS6KA2 and RPS6KA3), a vesicle docking protein (VDP) and two metallothioneins (MT1A and MT2A).

Interestingly, a third cluster (cluster 3) identified genes with opposite behavior at high and low Cu concentrations. In fact, in cluster 3 we found 164 genes up-regulated in cells treated with Cu 10^{-6} to 10^{-9} M, and down-regulated by Cu at lower concentrations (cluster 3a). There were genes groups codifying for an ATPase transporting protein (ATP5C1), members of the collagen family (COL12A1, COL4A3BP and COL8A1), histone proteins (H2BFS, HIST1H1D, HIST1H1E, HIST1H2BE, HIST1H2BM and HIST1H2BN), heat shock proteins (HSP90AB1 and HSPA8), metallothionein (MT1M), a metal-regulatory transcription factor (MTF1) and a chaperonin (HSP60).

A second group of 244 genes was down-regulated in cells treated with Cu 10^{-6} to 10^{-13} M and up-regulated at lower doses (cluster 3b). Among these genes we noted an ATPase transporter (ATP2C1), a lysyl oxidase (LOX), a metal ion transporter (SLC39A11) and other solute carriers (SLC44A2 and SLC7A11).

Finally, a third group of 233 genes were up-regulated up to 10^{-14} M and down-regulated from 10^{-15} to 10^{-17} M (cluster 3c).

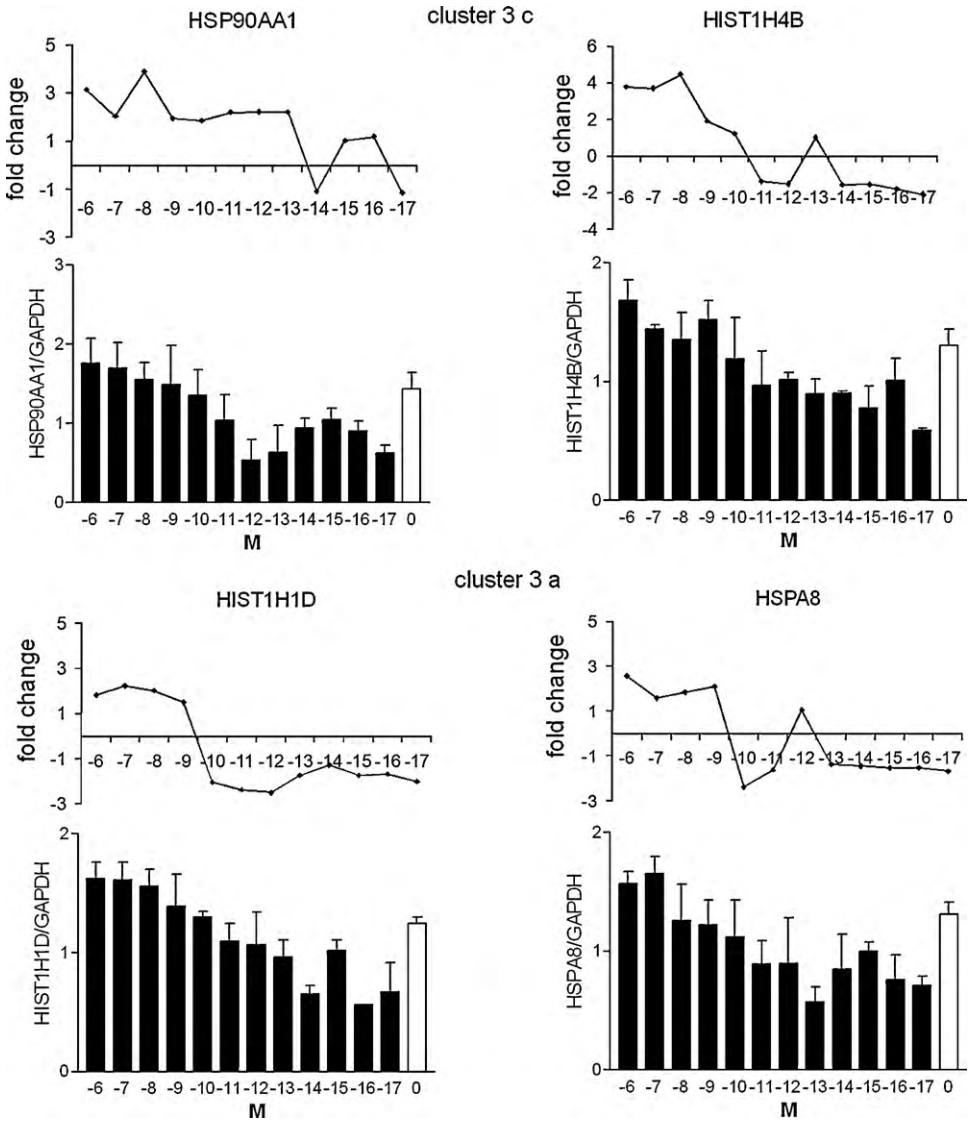

Fig. 3. Effects of variable Cu concentrations on the expression levels of four selected genes in five biological replicates. Bars represent means ± SD; *Y*-axis values are ratios between the expression of each gene relative to the co-amplified housekeeping gene (GAPDH); *X*-axis values are the Cu concentrations added to the cell culture medium. In the panels above each bar plot, we report the microarray profiles of the same gene, expressed as fold changes.

Among them, were a cytochrome c oxidase (COX15), genes codifying for histone proteins (HIST1H4B, HIST1H4C, HIST1H4L and HIST2H2AC), a heat shock protein (HSP90AA1), ribosomal proteins (MRPL21, MRPL3, MRPS36, RPL34, RPL39 and RRBP1), solute carriers (SLC25A24 and SLC43A2), ubiquitin-conjugating enzymes (UBE2O, UBPH and USP48) and metallothioneins (MT1B, MT1E, MT1G and MT1H).

The second, independent set of hybridizations, using pools of RNA from five biological replicates for each Cu concentration, mainly confirmed previous results, when analyzed with the K-means clustering analysis. It was possible to identify a cluster of genes down-regulated between 10^{-6} and 10^{-13} M and up-regulated at lower concentrations (Fig. 3, cluster 3b), a cluster of genes up-regulated from 10^{-6} and 10^{-13} M (Fig. 3, cluster 3c) and codifying for metallothioneins, ribosomal heat shock and histone proteins.

This last analysis also showed clusters of down- or up-regulated genes at all Cu concentrations; among these genes we found the same classes identified previously, such as ATPase transporting proteins, proteasome/ubiquitin system members and metallothioneins. Interestingly, among the most down-regulated genes at all Cu concentrations, we found genes encoding for keratins (K6HF, KRT23, KRT6L, KRT16, KRT13, KRT6E, MGC102966 and KRT34), known to be affected by a variety of stressors including Cu toxicosis [16], suggesting that even low amounts of Cu can induce cellular stress.

Using functional analysis we identified several pathways modulated by copper treatments (Table 1). Many pathways were statistically up or down-regulated; among others, prostaglandin synthesis/inflammatory response and TGF beta signaling pathways were up-regulated in cells treated with Cu compared to untreated cells. Accordingly, Cu chelators in various experimental models have been shown to reduce inflammatory cytokines and TNFbeta signaling [17–19]. The pathway of myosin/actin cytoskeleton was also consistently up-regulated. Heavy metals at high concentration often have an effect on the cytoskeleton [20]. Members of the myosin family are also involved in the transportation of vesicles and proteins, and their increased expression may reflect enhanced transport of Cu by intracellular vesicles. Genes involved in adipogenesis, Krebs cycle, Wint signaling and methionine metabolism were consistently down-regulated by Cu, whereas proteasome degradation and electron transport genes were down-regulated

Table 1
Results of the pathway analysis using GenMAPP/MAPPFinder software. Pathways significantly enriched (z score >1.97) in up- or down-regulated genes in cells treated with Cu solutions from 10^{-6} to 10^{-17} M, compared to untreated cells. The values in each box represent the number of genes significantly up- or down-regulated in each pathway.

	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}	10^{-14}	10^{-15}	10^{-16}	10^{-17}
Up-Regulated Pathways												
Inflammatory_Response/Prostaglandin synthesis_regulation	16	7	10	9		4	5	14	3	3		4
Focal_adhesion	23	17	32	35		17						
Myosin/actin cytoskeleton	7	7	7	9	6	4	4	8	11		4	
TGF_Beta_Signaling_Pathway	8	5	11	12	6		6		6		5	
Ribosomal_Proteins	25		23			15	11	20		51	20	50
Down-regulated pathways												
Cholesterol_Biosynthesis	9	5	11	7	6							
Notch_signaling	12	11	19		10			11				
TCA_cycle_	10	6	8				4	10			6	
Wnt_Signaling	23	16	23	14	7					16	12	
Methionine_metabolism	5	4	5		18			5		4		
Adipogenesis		19	22	22		9		19		17	15	
Proteasome_Degradation						10		16		14		
Electron_Transport_Chain									17			17
Ribosomal_Proteins						18	12	40				18

at the lower end of the concentrations tested and cholesterol biosynthesis genes between 10^{-6} and 10^{-10} M. A lowering of cholesterol biosynthesis is associated with copper overloading. Atp7b$^{-/-}$ mice, a model of Wilson disease, have down-regulation of cholesterol metabolism and decreased liver cholesterol [21]. Altered cholesterol homeostasis has also been reported in patients with Wilson disease [22]. Other literature data also suggest a role of Cu on adipogenesis; prolonged Cu deprivation in rats can induce islet hyperplasia and hepatic metaplasia in the pancreas and Cu re-feeding causes a replacement of fibroblasts with adipocytes [23].

It is interesting to observe that when testing Cu concentrations beyond the detention limit of current instrumentations, we identified modulations of genes or/and pathways which are altered also at toxic Cu concentrations. Accordingly, Muller et al. applied microarray analysis to the study of the expression profiles of HepG2 cells incubated with Cu 10^{-4} M at different times [24]. They observed an up-regulation of metallothioneins as an early response and, after 18–24 h, gene modulations associated with oxidative stress response, such as heat shock proteins and genes involved in ubiquitination and proteasome degradation [24]. Therefore, it appears that even the addition of femtomoles of Cu evokes cellular responses which are similar to those set in motion by toxic concentrations.

Reverse-transcription-PCR (RT-PCR) analysis was performed on five independent biological replicates for each Cu concentration, with the aim of evaluating the quantitative variation observed with the microarray experiments. For this analysis we selected four genes among those showing opposite behavior at different Cu concentrations (HIST1H1D, HIST1H4B, HSP90AA1 and HSPA8).

Fig. 4 shows the RT-PCR expression data of these genes. In the upper panels, for comparison, we reported the results obtained with microarray analysis. The trends in RT-PCR data and in the microarray analysis are similar in terms of gene expression.

Some of the effects of Cu on gene expression we describe might seem surprising when considering the small amounts of Cu added, often orders of magnitude lower than the total Cu present in the medium. We calculated that in RWPE cells the total amount of Cu, determined by plasma mass spectrometry on whole cell suspensions, was 2.3×10^{-15} moles per cell (1.39×10^{9} atoms per cell). In eukaryotic cells, the free Cu is less than 10^{-18} M, due to the extraordinarily restricted free Cu availability for extensive intracellular chelating activity [25]. Living organisms have complex systems for Cu trafficking to drive it to its cellular destination and limit toxicity, involving the interaction with proteins with very high copper affinity, with affinity constants such as the concentrations tested in our work [26].

Overall, our results suggest that Cu exposure may have biological effects even at extremely low concentrations and that measuring variations in gene expression may help to explore the biological effects of trace nutrients, contaminants or drug, at concentrations lower than the current limits of instrumental detection.

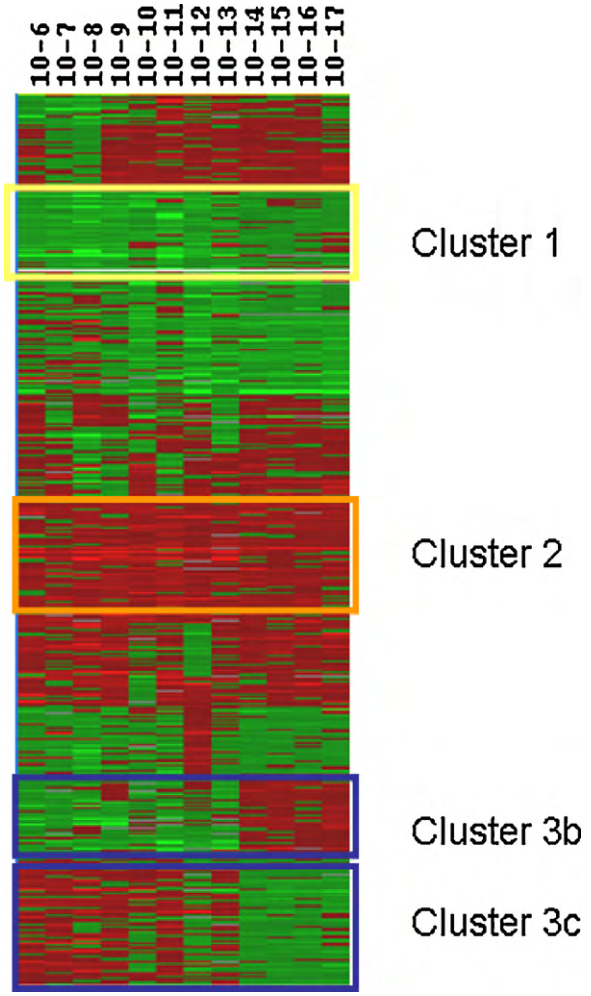

Fig. 4. K-means cluster analysis of microarray data obtained analyzing a pool of RNAs from five independent biological replicates of each Cu concentration.

Conflict of interest statement

The authors state that there are no conflicts of interest.

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