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Article

CRISP-R/Cas9 Mediated Deletion of Copper Transport Genes CTR1 and DMT1 in NSCLC Cell Line H1299. Biological and pharmacological consequences

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Abstract: Copper, the highly toxicity micronutrient, plays two essential roles: it is a catalytic and structural cofactor for Cu-dependent enzymes, and it acts as a secondary messenger. In the cells, copper is imported by CTR1, a transmembrane high-affinity copper importer, and DMT1 (divalent metal transporter). In cytosol, enzyme-specific chaperones receive copper from CTR1 C-terminus and deliver it to their apoenzymes. DMT1 cannot be a donor of catalytic copper because it does not have cytosol domain which is required for copper transfer to the Cu-chaperons and following to cuproenzymes. Here we assume that DMT1 can mediate copper way required for regulatory copper pool. To verify this thought, we used CRISPR/Cas9 to generate H1299 cell line with CTR1 or DMT1 single knockout (KO) and CTR1/DMT1 double knockout (DKO). To confirm KOs of the genes qRT-PCR were used. Two independent clones for each gene were selected for further studies. In CTR1-KO cells, expression of the DMT1 gene was significantly increased. In subcellular compartments, copper concentration decreased dramatically in DKO cells. CTR1-KO cells, but not DMT1-KO, demonstrated reduced sensitivity to cisplatin and silver ions, agents that enter the cell through CTR1. The expression of genes, whose protein products require copper: HIF1α, XIAP, COMMD1, CCS, Cp, but not SOD1 and NF-kB, changed their level. Perhaps these data will help to understand how the disturbances of copper homeodynamics lead to the development of neurodegenerative and oncological disorders. Possibility of using CTR1 KO and DMT1 KO cells to study homeodynamics of catalytic and signaling copper selectively is discussed.

Keywords: copper importers CTR1 and DMT1, CRICPR-Cas9, cisplatin, silver, signaling, copper homeodynamics

1. Introduction

In mammals, copper has two essential physiological functions. First, it is a catalytic and structural cofactor of enzymes necessary for respiration, antioxidant protection, post-translational modification of neuropeptides, for the synthesis of neurotransmitters, for the formation of collagen and elastin, for iron transport, etc. [1,2]. Second, copper inside and outside the cell is required for the activity of some regulatory proteins (HIF1α, XIAP, COMMD1, NF-kB, p53), which are involved in signaling pathways [3-7]. The indispensable biological functions of copper are inseparable from its

high toxicity, which is compensated by an intricated system of carriers that coordinate copper through specific sites, and safely transfer it from the extracellular space to the cell places of cuproenzyme formation (mitochondria, Golgi apparatus, cytosol) [8].

It is still unclear how copper is recruited into signaling pathways, or how it is secreted from the cell to participate in neovascularization [9]. As a non-sophisticated version of this issue, it can be assumed that these copper streams enter the cell through different gates. The main universal importer of copper in mammalian cells is CTR1 [10,11]. It is a transmembrane homotrimer, each subunit of which contains 3 α-helices, forming a transmembrane domain of 9 α- helixes. The N-terminal extracellular domain contains 3 Cu(II)/Cu (I) sites. In a homotrimer, N-termini form a high affinity copper trap from the environment, where its concentration is low. The short C-terminal domain contains a copper-binding HCH-motif. All cytosolic copper carriers required for cuproenzymes, taking delivered copper, due to a protein-protein interaction with C-domain CTR1 [12]. One might think that catalytic copper enters through the CTR1.

The cells contain another importer, also capable of carrying copper. It is the divalent metal transporter 1 (DMT1), also known as divalent cation transporter 1 (DCT1) and natural resistance-associated macrophage protein 2 (NRAMP 2), the member 2 of solute carrier family [13]. It consists of a single subunit. Putative topological DMT1 model predicts that DMT1 is a type III integral membrane protein (both N- and C-terminus are oriented to the cytoplasm), with transmembrane domain from 12 α- helixes and two sites for core N-glycosylation in the extracellular loop between 7 and 8 α- helixes [14]. It's well established that it functions as proton-coupled pump by using the cell membrane potential for active transport. DMT1 imports iron as Fe(II), and also Mn(II), Zn(II), Cu(II), Ni(II), Co(II), Pb(II) and Cd(II) ions; it is ubiquitously expressed, most notably in the apical membrane of the enterocytes in duodenum [13]. The cation binding peptide is formed by DMT1-α-helix1 as an α-helix-extended segment–α-helix configuration, in which the negative charged motif Asp-Pro-Gly-Asn responsible for cation binding is located at the central flexible region [15].

DMT1 transports copper in both Cu (II)/Cu (I) oxidation states [16], and even when the CTR1 gene is switched off, it compensates for its deficiency [17]. C-terminal domain of DMT1 has no homology with C-terminus of CTR1, so, it is unlikely that it is capable of transferring copper to cuproenzyme-associated Cu-transporters. It is yet not known which proteins, or low molecular weight substances, take copper from DMT1. It is possible that DMT1 represents the pathway for regulatory copper. To check this hypothesis, we obtained single knockout CTR1 and DMT1 cells as well as double knockout of both genes. Engineered cells were tested for resistance to cisplatin and silver ions, and to some additional drugs acting with different mechanisms of action. The expression profile of genes coding copper-requiring proteins was also determined.

2. Materials and Methods

2.1. Cell lines

The human non small cell lung cancer cell line H1299 was used for these experiments. These cells do not express the tumor suppressor p53 protein. Cells were grown in RPMI medium supplemented with 10% FBS. Cells were transfected with CRISPR-Cas9 KO plasmids with three targets specific guide RNAs (gRNA) of 20 nt (for both CTR1 and DMT1 genes). The plasmids were co-transfected with Homology-Directed Repair (HDR) plasmids specific for each gene (Santa Cruz Biotechnology), which allow the insertion of puromycin resistance gene and red fluorescent protein (RFP) gene during the repair process. Puromycin and RFP genes can then be removed using Cre recombinase, thanks to the presence of loxP sites in HDR plasmids.

Cells were seeded at high density 24 hours before transfection in 6 well plates. Fugene (promega) was used as transfection reagent. Twenty-four hours after transfection, cells were detached and seeded in 10 ml Petri dishes at a density of 500 cells /plate. After the following 48 hours, puromycin (2 ug/ml) was added to allow selection of positive clones. A double selection of clones growing in puromycin containing medium and expressing RFP was performed. Positive clones were isolated and transferred to 6 well plates.

Expression of CTR-1 or DMT-1 mRNA by RT-Real time PCR was used to confirm the KO of the genes. Two independent clones for each gene were selected for further studies.

For generation of double KO cells (for both CTR-1 and DMT-1 genes) one clone deleted in CTR-1 was treated with Cre recombinase to remove puromycin and RFP genes and subjected to a second round of transfection using a mix of DMT-1 KO plasmid and DMT-1 HDR plasmid using the same procedure as described for single KO generation.

2.2. RT-Real Time PCR

Total RNA was extracted from exponentially growing cells using Maxwell RSC simply RNA Cells kit (Promega) and reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies). DMT1, CTR1, XIAP, HIF1α, NF-kB, COMMD1, CCS, SOD1 expression levels were determined by real time RT-PCR performed with Sybr Green PCR master mix (Applied Biosystem). For each gene and each sample, the dissociation curve was evaluated. Samples were then normalized using the expression of the housekeeping gene (actin) and the levels in the KO clones were compared to parental cells. Real-time PCR was performed using the 7900HT Sequence Detection System (Applied Biosystems).

2.3. Cell growth and cytotoxicity

The growth in vitro of the different clones was determined using the RealTime GLO system (Promega). All the procedures were performed according to the manufacturing instructions. Luminescence was detected at 24 hours interval using the GloMax plate reader (Promega). For each clone 6 independent samples were assessed, and the mean doubling time calculated from the linear part of the growth curve for each cell line.

The growth inhibitory activity of two platinum containing drugs, cisplatin and carboplatin, of the mTOR inhibitor Torin-1, of the ATR inhibitor VE-822, of the GLUT-1 inhibitor STF-31and of the widely used antidiabetic drug metformin, was determined in the different clones by using the MTS test. Cells were seeded in 96 wells plates and after 24 hours treated with increasing concentrations of the drugs for further 72 hours. Survival curves were plotted as percentages of untreated controls. At least 6 replicates for each time point were used and the results represent the average mean and SD of at least 3 independent experiments.

2.4. Isolation of subcellular fractions

Subcellular fractions were isolated by differential centrifugation. Cells were homogenized (1:6 w/v, respectively) in buffer A, containing 250 mM sucrose, 100 mM KCl, 5 mM MgCl2, 10 mM Tris-HCl (pH 7.4), 5 mM DTT, and 0.5 µl/ml of protease inhibitor cocktail (Sigma, USA), using T10 basic homogenizer for 3×20 s at maximum power (IKA, Germany). The homogenate was centrifuged at 800×g for 10 min. A crude mitochondrial fraction was isolated from the post-nuclear supernatant as sediment after centrifugation at 12,000×g for 20 min. A total intracellular membrane fraction (endoplasmic reticulum + Golgi complex) was isolated from the post-mitochondrial supernatant as sediment at 23,000×g, 60 min. The supernatant of the last centrifugation comprised the cytosolic fraction.

2.5. Expression of Cp secreted in the medium was assessed by immunoblotting (WB) in samples of cell culture supernatants. Twenty-five microliters of cultured medium were separated on 8% polyacrylamide gels (PAG) by nondenaturated electrophoresis according to the Laemmli method. The protein transfer, control for the quality of transfer with PONCEAU staining, blocking with 5% non-fat milk, blotting with primary antibodies, and visualization of the immune complexes were described previously [18]. Hybond ECL nitrocellulose membrane, ECL reagent, ECL Hyperfilm (GE Healthcare, USA), and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Abcam, UK) were used for the WB analysis. In the work, non-commercial antibodies to high purity human ceruloplasmin were used [19].

2.6. Preparation the growth medium saturated with silver ions

The portions of AgCl crystals were added to RPMI medium with stirring until AgCl crystals ceased to dissolve. Ag-medium was clarified by centrifugation at 10000×g, 1 h. This medium was considered as saturated with Ag(I) growth medium, in which silver atoms are coordinated. Silver concentration in the Ag-saturated medium was determined by atomic absorption spectrometry. It was 150 μM.

2.7. Measurement of metal concentration

Copper and silver concentrations were measured by graphite furnace atomic absorption spectrometry (FAAS) with electrothermal atomization and Zeeman correction of nonselective absorption on a ZeeNit P650 spectrometer (Analytik Jena, Germany) with automatic sampling duplication. The samples were dissolved in pure concentrated HNO3.

2.8. Statistical analysis

The statistical analysis was performed using GraphpadPrism version 6.07. Differences between groups were considered statistically significant, when the p-values were ≤0.05.

3. Results

Using H1299 cells, we generated CTR1 and DMT1 single KO clones using CRISPR-Cas9 system. Two independent homozygous KO clones for each gene were selected (clone 45 and 46 for CTR1 and clones 20 and 31 for DMT1). From a CTR1 KO clone (clone 45) a second round of transfection, following excision of puromycin and RFP genes by Cre recombinase, to generate CTR-1/DMT1 double KO. These last clones were less efficiently generated compared to single KO genes. In fact, while we could isolate several CTR1-/- or DMT1-/- clones, we were only able to isolate one CTR1-/-, DMT1+/- (DKO 4) clone and one CTR1-/-, DMT1-/- clone only (DKO 5). The majority of the experiments were performed in one CTR1-/- clone, one DMT1-/- clone and one DKO clone, and some results were confirmed in the second generated clone for each genotype.

Selected clones were initially tested for their ability to grow in vitro. Table 1 reports the doubling times (DT) calculated on three independent experiments, each consisting of six replicates. As it can be seen the selected clones have similar DT all growing slightly faster than parental H1299 cells.

Table 1. Doubling time of H1299-derive clones

CELL LINE	DOUBLING TIME, (h) ±SD
H1299	18.77±1.49
CTR1 KO45	15.03±4.09
DMT1 KO31	13.45±2.31
DKO 4 ($CTR1^{-/-}$, $DMT1^{+/-}$)	14.51±3.30
DKO 5 ($CTR1^{-/-}$, $DMT1^{-/-}$)	15.36±2.56

Figure 1 displays the estimates of expression of DMT1 and CTR1 by RT-Real time PCR in parental H1299 cells, in one clone KO for CTR1 (clone 45), one clone KO for DMT1 (clone 31), one clone CTR1-/-, DMT1+/- (DKO 4), and one clone CTR1-/-, DMT1-/- (DKO 5).

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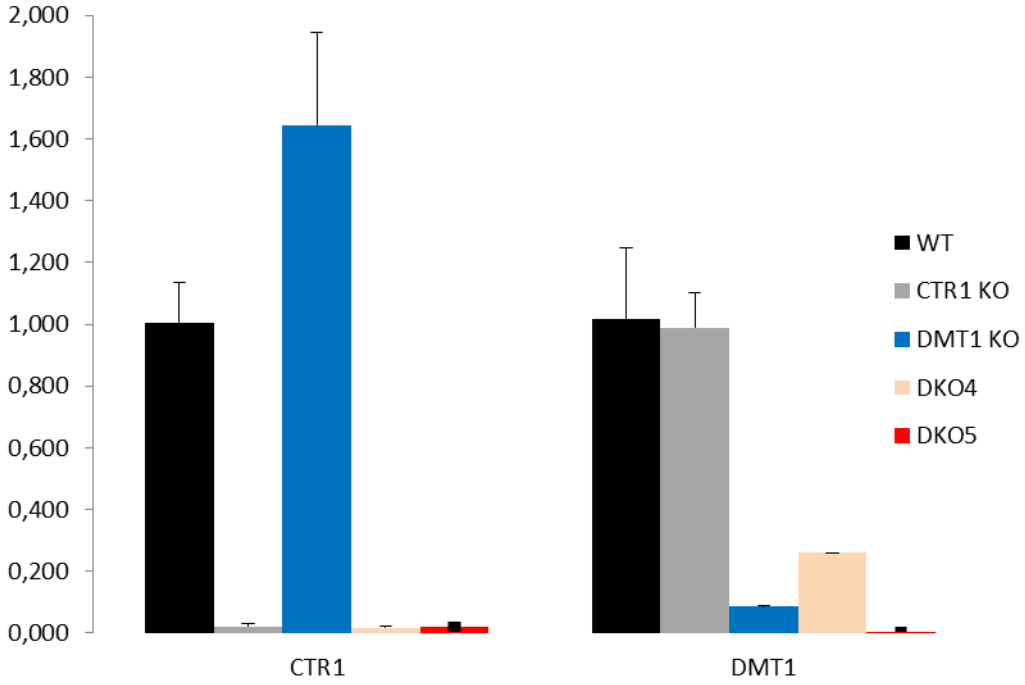

Figure 1. RT-Real time PCR analysis of CTR1 and DMT1 mRNA expression in the different clones. Ordinate: concentration mRNA of CTR1 or DMT1 to actin-mRNA.

A clear lack of expression of the respective target genes is seen for single KO cells. DKO 5 clone showed a dual inhibition of expression, and DKO 4 clone, which is DMT1+/- heterozygous clone, expresses DMT1 2 times higher than DMT1-/-. Interestingly CTR1 KO clone showed a consistent increase in DMT1 expression, while in the DMT1 KO clone the expression of CTR1 was similar to that of parental cells.

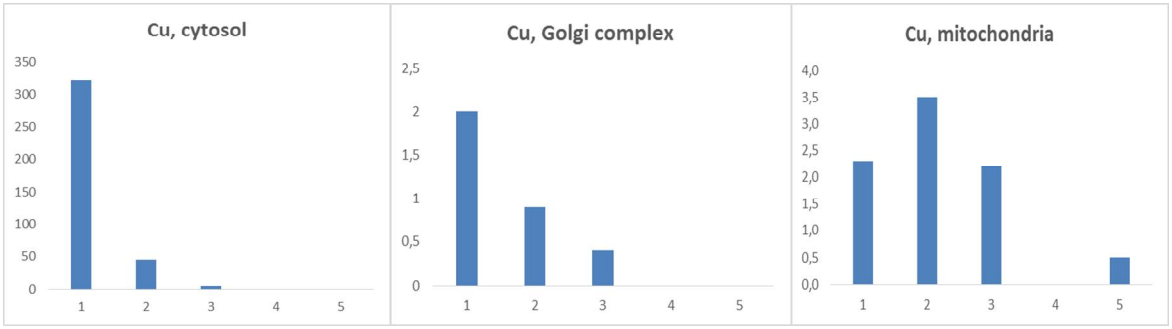

Figure 2. Copper distribution in CTR1 KO, DMT1 KO and DKO cells. Ordinate: copper concentration (μg/L) in subcellular fractions. 1 – control, 2 – CTR1/45, 3 – DMT1/31, 4 – DKO5, 5 – DKO4.

The effects of the single and double knockout of CTR1 and DMT1 on copper distribution in the cells are shown in Figure 2. In cytosol of CTR1 KO/45, the copper level decreased by a factor of 6. In DMT KO clone, the copper content was even lower, and in DKO, the content of copper dropped further below the threshold of reliable measurements. In the Golgi apparatus, the copper level in CTR1-KO/45 decreased almost twofold, in DMT KO/31, it was roughly 1/8 of that of parental cells while in the two DKO clones the levels of copper in Golgi were below the detection level. In the mitochondria of cells with single knockouts, the copper content remained almost unchanged, but it sharply decreased in both DKO cells. The cells were tested for the toxic effect of silver ions. Figure 3 demonstrates that CTR1-KO clones are more resistant to silver ions than wild-type (WT) cells, or DMT1-KO clones.

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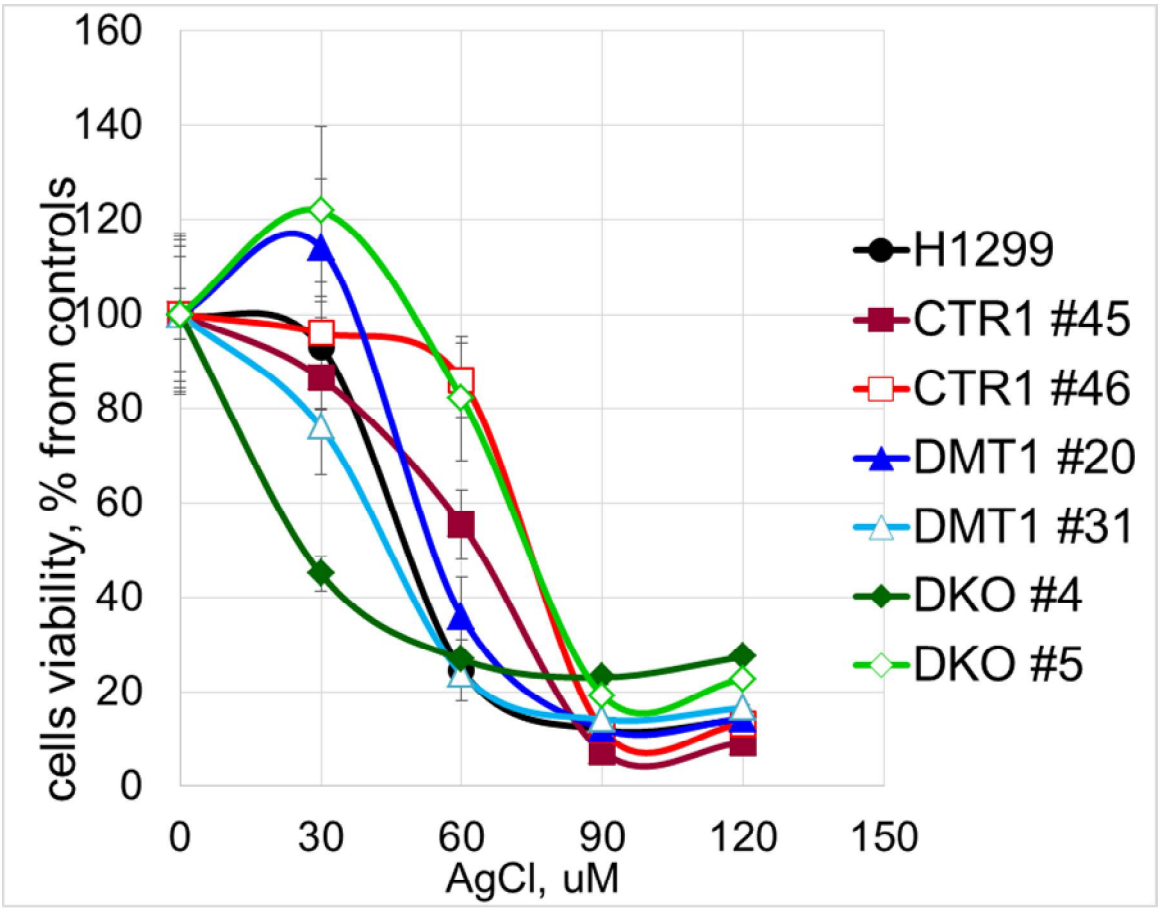

Figure 3. Cells viability after treatment in medium with AgCl for 72 hours.

In these clones, the distribution of silver was traced at different concentrations in medium (Figure 4). In the cytosol, the Golgi complex membranes and in the mitochondria, in DMT1-KO clones, silver content was about the same as in WT cells. At the same time in CTR1-KO and DKO clones compartments, the silver concentration was 3 to 5 times lower.

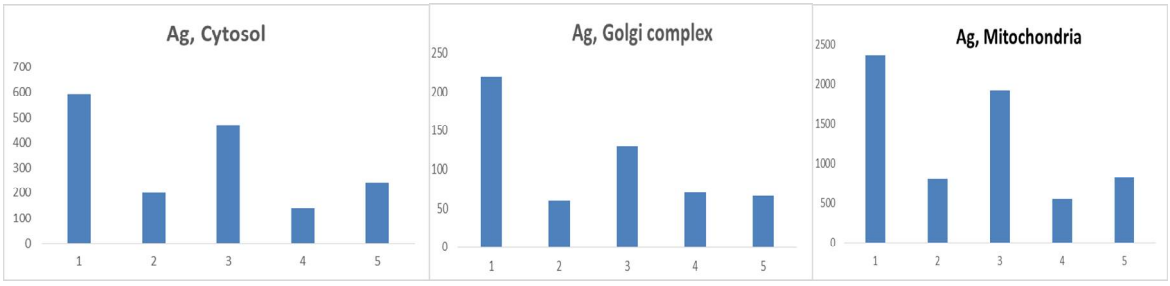

Figure 4. Silver distribution in CTR1 KO, DMT1 KO and DKO cells. Ordinate: silver concentration (μg/L) in subcellular fractions. 1 – control, 2 – CTR1/45, 3 – DMT1/31, 4 – DKO5, 5 – DKO4.

Using the example of ceruloplasmin, we checked the ability of the obtained clones to synthesize secretory cuproenzymes. Ceruloplasmin, a polyfunctional blue (ferr)oxidase of hepatic origin, is the main copper-containing blood serum protein [20-22]. It has been shown that ceruloplasmin is also synthesized in the lungs [23], its function has not been established. We first checked whether fetal calf serum (FSB) will give false positive signals in WB analysis. The data in Figure 5A shows that FSB does not give a positive signal with antibodies to human ceruloplasmin. WT H1299 cells synthesize and secrete ceruloplasmin (Figure 5A), which by mobility corresponds to holo-ceruloplasmin. In CTR1-KO, DMT1-KO and DKO cells, the synthesis of immunoreactive ceruloplasmin decreased

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(Figure 5C). Moreover, in all KO clones, ceruloplasmin was detected as two bands that correspond to holo- and apo-ceruloplasmin [24].

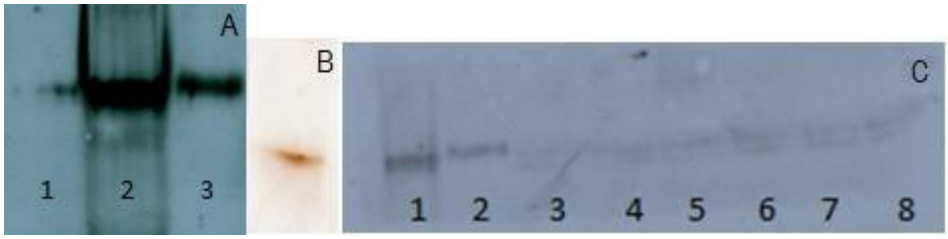

Figure 5. Ceruloplasmin gene activity in CTR1-KO, DMT1 KO, and DKO. **(A)** WB of 8% non-denaturated PAGE, 1 – FSB, 0.5 µl per line; 2 – human serum, 0.025 µl per line; 3 – H1299 medium of cultivation, 25 µl per line. **(B)** human serum, 0.025 µl per line, gel was stained with orto-dianisidine; (C) 1 – human serum, 0.025 µl per line; 2 – H1299, 3 – CTR1 45, 4 – CTR1 46, 5 – DMT1 31, 6 – DMT1 20, 7 – DKO 4, 8 – DKO 5, 25 µl medium per line

We next evaluated the pattern of gene expression for selected genes known to be linked to copper transport and more in general to copper homeostasis. In particular we tested at basal condition the expression of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), HIF1alpha (hypoxia-inducible factor 1-alpha), SOD1 (Cu,Zn-superoxid dismutase), XIAP (X-linked inhibitor of apoptosis protein), COMMD1 (copper metabolism domain containing 1) and CCS (copper chaperone for superoxide dismutase). The results are shown in Figure 6.

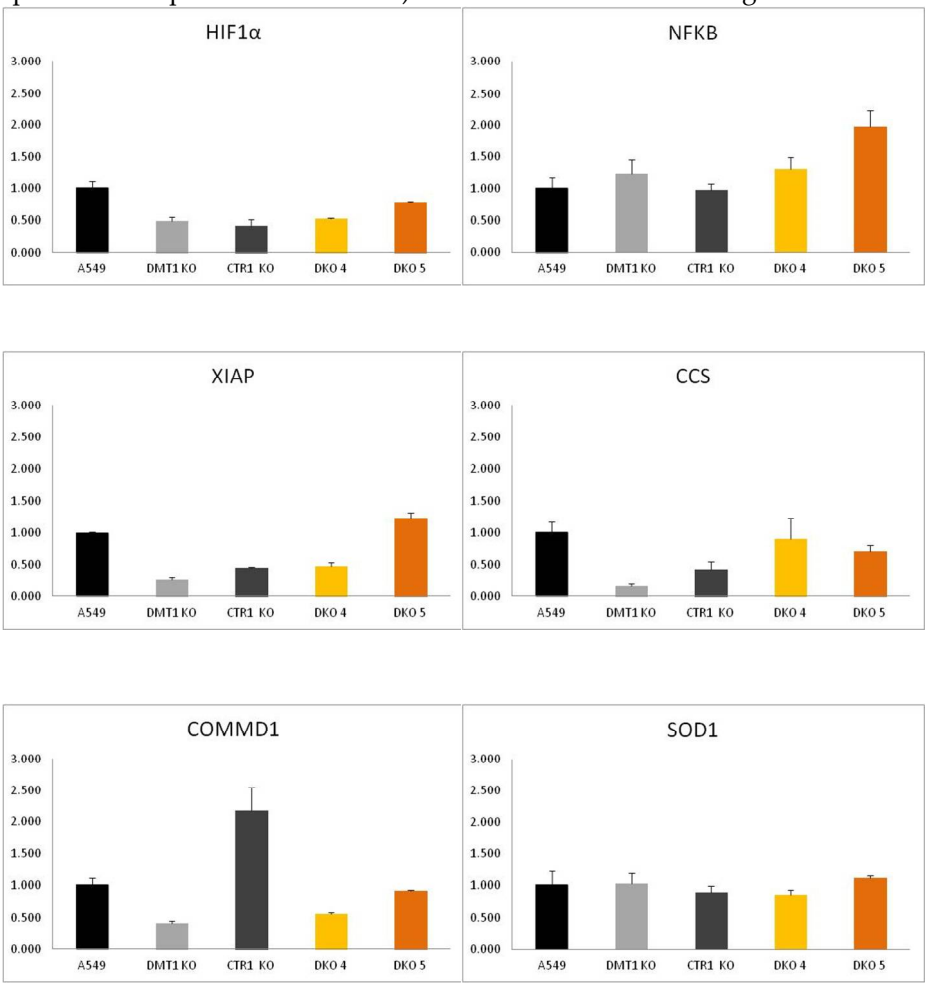

Figure 6. RT Real time expression PCR analysis of the mature mRNA concentration, which are the products genes related copper metabolism, in the different H1299-derived clones.

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In DMT1-KO cells, the expression of the HIF1, COMMD1, XIAP, and CCS genes is reduced by more than 2 times while NF-kB and SOD1 levels were almost unchanged. In CTR1-KO cells, a decrease in HIF1, XIAP and CCS levels was also observed, but in contrast the expression of COMMD1 gene was significantly increased, while NF-kB and SOD1 gene expression did not change as in DMT-1 KO clones. DKO clones showed a mixed response, being HIF1the only gene whose expression was convincingly reduced. The expression of the other genes was either only slightly decreased or remained unchanged.

Finally the clones were tested for their ability to respond to cytotoxic compounds, including cisplatin (known substrates of CTR1 [11]), carboplatin (cisplatin analog), metformin, VE-822, Torin-1 and STF-31. The obtained results are presented in Figure 7.

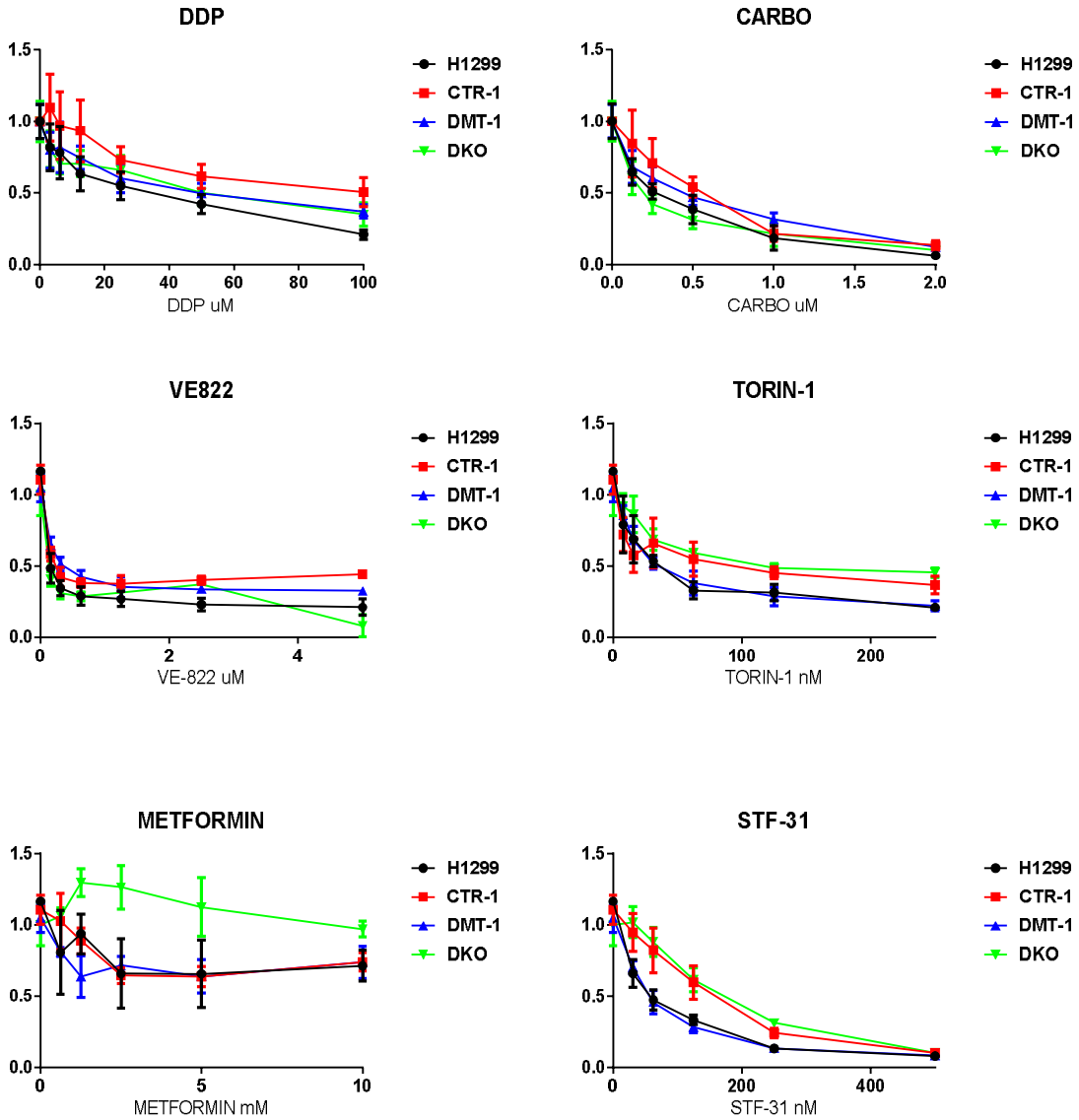

Figure 7. Cytotoxic activity of cisplatin, carboplatin, VE-822, Torin-1, Metformin and STF-3 in H1299 parental cells and in KO derived. Abscess: drug concentrations, μM, Abscess: drug concentrations. Ordinate: growth rate. Growth inhibitory activity was determined using the MTS test. Each point represents the mean ±SD of six replicates.

CTR1 KO cells were significantly more resistant to cisplatin then parental H1299 cells. The other clones displayed cisplatin sensitivity similar to parental cells. Carboplatin showed similar activity in parental and KO cells. VE-822, an inhibitor of the stress response ATR signaling pathway in DNA repair [25]; TORIN-1, mTOR inhibitor, a member of phosphatidylinositol 3-kinase-related kinases

family, which functions as a serine/threonine protein kinase in different signaling way [26]; displayed similar toxicity for all the clones. STF-31, specific glucose transporter 1 inhibitor [27], showed a slightly reduced activity in CTR-1 KO cells and even less in DKO clone. Meanwhile, interesting results were obtained with the AMPK inhibitor metformin, which decreases blood glucose concentration and is widely used in the treatment of obesity and now largely in some types of cancer [28]. With this drug parental cells and the single KO (both CTR-1 and DMT-1) behaved similarly. The both DKO clones were instead much less responsive.

4. Discussion

The aim of this study was to generate and characterize the CTR1 and DMT1 single knockout and double CTR1/DMT1 knockout clones and to evaluate them as potential model for the studies of copper metabolism in catalytic and regulatory copper pools. The results will be mostly discussed with respect to the latter purpose. Doubling time of single KO clones derived from H1299 was similar to that of parent cells. Therefore, these cells are convenient for laboratory studies. As expected, only traces of CTR1-mRNA were detected in CTR1 KO/45 clone (Figure 1). Meanwhile, concentration of DMT1-mRNA increased by a factor of 1.6, generally agreeing with the observation that DMT1 can compensate the loss of CTR1 function and restore copper balance in the cell [16]. However, in DMT1-KO clone the concentration of CTR1-mRNA did not change, thus suggesting that the compensation is gene-specific. In DKO clones, both CTR1 and DMT1 levels were almost absent, except for DMT-1 in DKO clone (heterozygous for DMT1) in which some DMT1 expression was detectable.

The loss of function of CTR1 or DMT1 genes in single KO clones led to copper deficiency in the cells and changed intracellular copper distribution (Figure 2). The copper levels in cytosol were strongly decreased as compared to parent line, but they were higher in CTR1 KO cells than in DMT1 KO cells, again in line with the ability of DMT1 gene to compensate CTR1 function [16]. It also indicates that CTR1 gene cannot equally compensate loss of DMT1 function (Figure 1). It is likely that the decrease of copper levels in cytosol corresponds to the depletion of Cu-metallothionein [29]. In mitochondria, copper concentrations did not significantly change as compared to parent line, in DMT1 KO cells its levels were even higher than in the wild type (Figure 2). It is possible that DMT1 completely satisfies the copper requirements of mitochondria, maintaining the proper level of vitally important mitochondrial cytochrome-c-oxidase. In Golgi complex copper concentration decreased by a factor of 2 and 4 in CTR1 KO and DMT1 KO clones, respectively (Figure 2). In DKO5 homozygous double knockout clone concentration of copper in all the isolated cellular fraction dropped below the used limit of detection. Copper could be detected only in mitochondria of DMT1-heterozygous clone DKO4.

We then used silver ions to compare the metal-transporting properties of CTR1 and DMT1 and to evaluate the difference between Cu(I) and Cu(II) transport routes. Cu(I) and Ag(I) have similar ion radii and the same structure of valence shells, so abiogenic Ag(I) is captured by Cu(I)-binding sites in CTR1 N-terminal domain and effectively transported into the cell. Accumulation of silver ions eventually leads to cell death [30]. Unlike CTR1, DMT1 does not contain thioether-rich metal-binding sites, so we supposed that CTR1-negative clones (CTR1 KO/45, CTR1 KO/46 and homozygous DKO5) would display increased resistance to silver ions as compared to wild type cells. Indeed, the cells that did not express CTR1 were more resistant to toxic effects of silver ions (Figure 3). Still DKO5 clone was the most resistant, indirectly proving that DMT1 can transfer both, Cu(II) and Cu(I)/Ag(I) [16].

The distribution of silver in the cells (Figure 4) of the parent line was in good agreement with known effects of Ag(I) binding by metallothionein [31], its accumulation in mitochondria [32] and its translocation to the lumen of Golgi complex [33]. We also paid attention that in the obtained clones, copper/silver is translocated to Golgi lumen (where secreted cuproenzymes are metallated) even in the conditions of copper deficiency. The loss of either CTR1 or DMT1 functions leads to the decrease in secretory ceruloplasmin synthesis and impairment of its metallation (apo-ceruloplasmin appeared in the secretion products) (Figure 5). It is established that the insertion of copper to

ceruloplasmin occurs in Golgi lumen and is catalyzed by copper-transporting P1 type ATPase (ATP7B). The copper is provided to ceruloplasmin by the following pathway: CTR1 → ATOX1 → ATP7B → ceruloplasmin [34]. Our present data indicate that copper atoms can be transported to Golgi lumen irrespectively from the import pathway.

The expression levels of genes, whose activity is linked or supposed to be linked to copper levels, were analyzed in the cells with the loss of CTR1 and/or DMT1 function. The results show that the genes under study were regulated differently (Figure 6). So SOD1 gene expression was not affected by CTR1 and/or DMT1 KO and was like that wild type cells.

Meanwhile, the activity of CCS gene coding Cu(I) chaperone for SOD1 decreased in single KO cells, but increased in DKO cells, in which copper cytosol concentration dropped below detection level. It is possible that in copper-deficient conditions apo-SOD1 is metallated in mitochondria [35], so the vital antioxidant function of holo-SOD1 is preserved. Thus, CCS gene may be activated when mitochondria use up all the copper that is not a part of cytochrome-c-oxidase pathway. However, the present data are insufficient to make reliable conclusions.

The decrease in expression of HIF1α and XIAP genes occurred both in CTR1 and DMT1 single KO lines, but the cytosol copper deficiency, which was characteristic of DKO lines, may activate the expression if these genes, as well as the expression of NF-kB gene. It is possible that these genes were activated by indirect signals, rather than directly by copper deficiency.

The changes of COMMD1 expression levels observed in the different clones are the most interesting. COMMD1 expression was enhanced in CTR1-deficient cells and decreased in cells with DMT1-deficient cells. COMMD1 is currently the only member of intracellular copper metabolic system that is not an oxidoreductase, but binds copper in Cu(II) oxidation state [36]. COMMD1 possesses sites for interaction with other copper transporters and may be viewed as a moonlighting protein [5]. Possibly, in wild type cells, COMMD1 received copper from DMT1 pathway, when DMT1 is knocked out and no copper is present in DMT1 pathway, COMMD1 gene expression decreases. In copper deficiency induced by the loss of CTR1 function, the role of COMMD1 in copper metabolism increases. Even if this is an attractive hypothesis, does not completely explain the levels of COMMD1-mRNA in DKO clones, which are similar to the levels present in the wild type cells. This could be the result of the sum of the effects of DMT1 KO (inducing a decrease of COMMD1 mRNA levels) and CTR1 (which instead increases COMMD1 expression) although this remains at present a speculation.

Tests for sensitivity of the obtained cells to various drugs (Figure 7) proved the fact, that the loss of CTR1, but not DMT1 rendered the cells resistant to cisplatin [12]. Other drugs, which were tested in the present work, affected the viability of the CTR1 KO, DMT1 KO, DKO and WT cells in similar manner. The only clear exception was metformin: DKO5 cells displayed resistance when exposed to this drug. Metformin has very diverse spectrum of interactions [28], including the ability to bind and transfer copper atoms [37], the latter may enhance the viability of copper deficient DKO5 cells. Being metformin widely used for different indication, including cancer, the knowledge that copper levels or transport can modify its activity could have, if confirmed important implications.

In summary the results obtained in the present work represents a good evidence that isogenic cell lines with differential knockouts of copper transporters and discriminated copper import pathways will be useful for studies of regulatory role of copper and could also have important translational relevance.

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