Roles of copper chaperone for superoxide dismutase 1 and metallothionein in copper homeostasis

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Copper chaperone for SOD1 (CCS) specifically delivers copper (Cu) to copper, zinc superoxide dismutase (SOD1) in cytoplasm of mammalian cells. In the present study, small interfering RNA (siRNA) targeting CCS was introduced into metallothionein-knockout mouse fibroblasts (MT-KO cells) and their wild type cells (MT-WT cells) to reveal the interactive role of CCS with other Cu-regulating proteins, in particular, MT. CCS knockdown significantly decreased Ctr1, a Cu influx transporter, mRNA expression. On the other hand, Atp7a, a Cu efflux transporter, mRNA expression was increased 3.0 and 2.5 times higher than those of the control in MT-WT and MT-KO cells. These responses of Cu-regulating genes to the CCS knockdown reflected the presence of excess Cu in the cells. To evaluate the Atp7a function in the Cu-replete cells, siRNA of Atp7a and the other Cu transporter, Atp7b were introduced into MT-WT and MT-KO cells. The Atp7a knockdown significantly increased the intracellular Cu concentration, whereas the Atp7b knockdown had no affect. Although two MT isoforms were induced by the CCS knockdown in MT-WT cells, the expression and activity of SOD1 were maintained in both MT-WT and MT-KO cells even when CCS protein expression was reduced to 0.30–0.35 of control. This suggests that the amount of CCS protein exceeds that required to supply Cu to SOD1 in the cells. Further, the CCS knockdown induces Cu accumulation in cells, however, the Cu accumulation is ameliorated by the MT induction, the decrease of Ctr1 expression and the increase of Atp7a expression to maintain Cu homeostasis.

Introduction

Copper (Cu) is an essential trace element in all living organisms. It forms the active center of cuproenzymes, such as Cu, zinc (Zn)-superoxide dismutase (Cu, Zn-SOD, SOD1), cytochrome c oxidase (CCO), ceruloplasmin, lysyl oxidase, tyrosinase, and dopamine b-hydroxylase.1 The function of Cu in the cuproenzymes is to facilitate the transition between cuprous (monovalent) and cupric (divalent) oxidation states. Harmful reactive oxygen species may be generated if free Cu ion exists in cells by this chemical property. Therefore, cells have strictly controlled mechanisms for the incorporation, intracellular distribution, utilization, and excretion of Cu. The mechanisms underlying Cu distribution to these cuproenzymes are suggested as follows.2 Cu is mainly incorporated into cells as cuprous ion by Ctr1, a transporter expressed on the plasma membrane.3,4 The incorporated Cu associates with one of three cytoplasmic Cu escort proteins, the so-called Cu chaperones, for distribution to specific organelles or cuproenzymes. It is known that the Cu chaperone for SOD1 (abbreviated as CCS) hands over Cu to SOD1 in cytosol by forming a heterodimer with SOD1. Indeed, CCS-knockout mice showed similar clinical manifestations to SOD1-knockout mice such as decreased SOD1 activity and enhanced sensitivity to oxidative stress. However, the CCS-KO mice SOD1 activity remains at 10–20% in all tissues except the liver, and 30% in the liver in spite of the lack of CCS.5 This suggests that SOD1 requires Cu supplementation by not only CCS but also the other Cu binding proteins to display its activity. The second Cu chaperone, Atox1, delivers Cu to Atp7a and Atp7b, which are ATP-dependent Cu transporters into the secretory pathway of Cu via the Golgi apparatus.6,7 In the Golgi apparatus, Cu is incorporated into such cuproenzymes as ceruloplasmin and lysyl oxidase, due to the secretion of these cuproenzymes into the extracellular fluid. Otherwise, Cu is directly effluxed from the cells by secretory vesicles that translocate from the trans Golgi membrane to the plasma membrane to excrete Cu. The third Cu chaperone, Cox17, a Cu chaperone for mitochondria, is required to donate Cu to CCO and/or SCO1, a recipient protein of Cu on the mitochondrial membrane.8,9 In addition to these Cu chaperones, metallothionein (MT) is suggested to be also a Cu-regulating protein. MT is a cysteinerich low molecular weight protein. Because it possesses abundant mercaptide bonds, MT actually binds Cu via Cu-thiolate clusters.10 As the binding of Cu by MT is thermodynamically and kinetically stable, excess Cu is sequestered by MT to mask Cu toxicity.11 On the other hand, an alleviative role of MT in Cu deficiency was also suggested.12,13 In our previous work, we noted that MT-null cells established from MT-knockout mouse fibroblasts (MT-KO cells) were more sensitive to the Cu-deficient condition induced by a cuprous-ion-specific chelator, bathocuproine sulfonate (BCS), than MT-wild type (MT-WT) cells.12 Thus,MT may play a dual role in maintaining Cu homeostasis in mammalian cells under both excess Cu and Cu-deficient conditions. As mentioned above, the functions of individual Curegulating proteins, such as membrane transporters, chaperones in the cytoplasm, and MT, have been clarified.

However, the interactive roles, i.e., crosstalk among Cu-regulating proteins, remain unclear. For instance, as regards the direct interaction between MT and SOD1, it was revealed that MT was able to directly donate Cu to SOD1 in vitro, similar to CCS.14 Atox1 knockdown induced Cu accumulation in the cells and the accumulated Cu could be detoxified by cytoplasmic vesicles in place of MT in MT-KO cells.15 Although Cu was accumulated by the Atox1 knockdown in both MT-WT and MT-KO cells, cells bearing Atox1 knockdown and MT-null mutation showed responses to Cu deficiency, such as decreases in Ctr1expression and Atp7b expression.16 This phenomenon could be explained as follows: elevated Cu was detoxified by compartmentalization and Cu compartmentalized in the vesicles might be less bioavailable than Cu bound to MT. These observations suggest that Cu-regulating proteins cooperatively and strictly regulate Cu homeostasis. In the present study, we focus on the interactive roles of CCS with other Cu-regulating proteins, in particular, MT. Small interfering (siRNA) targeting CCS was introduced into MT-WT and MT-KO cells and the expression of Cu-regulating factors, SOD1 activity, and the content and distribution of Cu were determined. In addition, although MT-WT and MT-KO cells established from fibroblasts of MT-WT and MT-null mice, respectively, express both Atp7a and Atp7b, it is still unclear why two functionally similar Cu transporters are expressed in the cells. Hence, the differences between Atp7a and Atp7b in the contribution to the Cu efflux were quantitatively clarified by the Cu determination in either Atp7a or Atp7b knockdown cells by an inductively coupled plasma mass spectrometry (ICP-MS).

Materials and methods

Cell culture

MT-WT and MT-KO cells were established from embryonic fibroblasts of 129Sv MT-WT and MT-KO mice transformed with SV40 large T antigen by Kondo et al.17 and kindly provided by Professor Seiichiro Himeno (Tokushima Bunri University, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) of the high glucose type (4500 mg/L) and supplemented with 10% heat inactivated fetal bovine serum, 10 U / mL penicillin, and 100 mg/mL streptomycin at 37 ˚C under 5% CO2 atmosphere.

Gene knockdown

Double-stranded RNAs (dsRNAs) were used as siRNAs (Stealtht RNAi, Invitrogen). The targeted sequence of CCS (50–UACUGAUGCACAUGGAGUCCAUGCA–30), Atp7a (50–UACCAAUGAGGACUUUGUACUGCUG–30) andAtp7b (50–CGUCUGUCAUGAACCUGCAGCAGAU–30) were designed from the mouse CCS (Genbankt accession number NM_016892), Atp7a (NM_009726), Atp7b (NM_007511) genes sequence. MT-WT and MT-KO cells were seeded on a 6-well plastic plate at 2.0 x 105 cells/well and pre-incubated for 24 h. The pre-incubated cells were transfected with 100 nM siRNA targeting CCS or control siRNA in the medium optimized for siRNA transfection (Opti-MEMsI, Invitrogen) containing 1.0% Lipofectaminet 2000 (Invitrogen) for 24 h.

Western blotting

Cells were lysed in phosphate buffered saline (PBS) containing 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and a cocktail of protease inhibitors (Hoffmann-La Roche Ltd., Basel, Switzerland) for 1 h on ice. The supernatant was obtained by centrifugation of the lysate for 20 min at 16 000 g at 4 1C.17 The protein samples were electrophoresed through 12.5% polyacrylamide gel and transferred to a PVDF membrane at 20 V for 20 min. The membrane was blocked overnight with 5% skim milk in 25 mM Tris-HCl containing 0.9% NaCl and 0.05% Tween 20, pH 7.5 (TBS-T) at 4 1C, incubated with anti-CCS (1 : 2500 diluted) or anti-SOD1 (1 : 2000) in TBS-T for 1 h, and washed three times with TBS-T. A synthetic peptide of the mouse CCS sequence H2N–WEERGRPIAGQGRKDS–COOH was used as an antigen to produce an antibody in three rabbits, and the antibody was purified by affinity chromatography using antigen-conjugated resin (Sawady Technology, Inc., Tokyo, Japan). Rabbit polyclonal antibody anti-SOD1 was purchased from Assay Designs, Inc. (Ann Arbor, MI, USA). The secondary antibody (1 : 2500) was incubated with the membrane in TBS-T containing 2% skim milk, and washed six times with TBS-T. This rabbit and mouse secondary antibody was purchased from GE Healthcare Bio-Sciences (Tokyo). The

blots were detected with an Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA) and analyzed with a Lumino-image analyzer LAS-1000 plus (Fujifilm, Tokyo) according to the manufacturer's instructions.

Assay for SOD1 activity

Cells were suspended in 25 mM Tris-HCl buffer containing 1% Triton X-100, 50 mM bathocuproinedisulfonic acid, 1 mM EDTA, and a cocktail of protease inhibitors at pH 6.8, and then sonicated for 5 min. The supernatant was obtained by centrifugation of the lysate for 20 min at 16 000 g at 4 ˚C. The protein samples were electrophoresed through 12.5% non-denaturing polyacrylamide gel. Then, the gel was stained with nitroblue tetrazolium (NBT) to detect SOD activity.18 SOD activity was detected as a decolorized band on the gel. The band was scanned and its digital image was quantified by NIH Image J 1.42 software. The assay was performed in three independent samples in each experimental group.

Real-time PCR

Total cellular RNA was extracted from MT-WT and MT-KO cells using Isogen (Nippon Gene, Tokyo). cDNA was synthesized from 0.2 mg of total RNA with a QuantiTect Reverse Transcription Kit (Qiagen, Tokyo). Amplification reactions were performed with SYBRs Premix Ex Taqt II (Takara, Shiga, Japan). Gene-specific primers used for amplification of mouse CCS, Ctr1 (NM_175090), Atox1 (NM_009720), Cox17 (NM_017429), SOD1 (NM_011434), MT-I (NM_013602), MT-II (NM_008630), Atp7a, Atp7b and b-actin (NM_007393) cDNAs were as follows: CCS-forward (forward, F), 5–CGGCCTAGGCAGTGACAACA– 3; CCS-reverse (reverse, R), 5–AGTCGTCTGCACCAACACCATC– 3; Ctr1-(F), 5–GACCACCTCAGCCTCACACT– 3; Ctr1-(R), 5–GGCATGGAATTGTAGCGAAT– 3; Atox1-(F), 5–TCAACAAGCTGGGAGGAGTG– 3; Atox1-(R), 5–ACATGGAAGCTTGCAGGGAG– 3; Cox17-(F), 5–TAGTCGGAGTTTGGGAGCTT–3; Cox17-(R), 5–ATTCACAAAGTAGGCCACCAC–3; SOD1-(F), 5–AGCATTCCATCATTGGCCGTA–3; SOD1-(R), 5–TTTCCACCTTTGCCCAAGTCA– 3; MT-I-(F), 5–CCTCTAAGCGTCACCACGACTTC– 3; MT-I-(R), 5–GGAGGTGCACTTGCAGTTCTTG– 3; MT-II-(F), 5–GCCTGCAAATGCAAACAATG– 3; MT-II-(R), 5–GCACAGCAGCTGCACTTGTC– 3; Atp7a-(F), 5–TGGTAACCGGGAATGGATGATTAG– 3; Atp7a-(R), 5–AACTCGGCCTCAGGTTTCACAG– 3; Atp7b-(F), 5–CAGCCAGAGCCATTGCTACTCA– 3; Atp7b-(R), 5–GAAGGCAGTACCTCCGCAAAGA– 3; b-actin-(F), 5–CATCCGTAAAGACCTCTATGCCAAC– 3; b-actin-(R), 5–ATGGAGCCACCGATCCACA– 3. Samples were analyzed in triplicate in a total volume of 50 mL using an ABI PRISM 7000 Sequence Detection

System (Applied Biosystems, Foster City, CA). b-Actin was used as the internal control for RNA quantification. RT-PCR was conducted under the following conditions: reverse transcription reaction of cDNA at 42 ˚C for 15 min, denaturation with reverse transcriptase at 95 ˚C for 3 min, followed by 40 cycles of PCR, i.e., denaturation of cDNA at 95 ˚C for 15 s and annealing and extension at 60 ˚C for 1 min.

Determination and speciation of Cu in cells

MT-WT and MT-KO cells were seeded and treated with CCS-specific or control siRNA according to the same protocol as that mentioned above. After the treatment, the cells were collected, and the harvested cells were wet-ashed with nitric acid (analytical grade, Wako, Osaka, Japan) on a hot plate and then diluted with deionized water. Cu concentration in the samples was determined with an ICP-MS (Agilent7500ce, Agilent Technologies, Hachiouji, Japan) at m/z 65. The sample was pneumatically introduced into the ICP-MS through an interface consisting of a micro flow nebulizer (PFA-20, Glass Expansion, West Melbourne, Australia) coupled with a cyclone chamber (Glass Expansion). For the speciation of Cu and Zn, the cells were collected and suspended to a concentration of 2.0 x 104 cells / mL with 10 mM Tris–HCl, pH 7.2. The suspended cells were disrupted with an ultrasonic homogenizer (Bioruptors UCD-200, Cosmo Bio Ltd., Tokyo) on ice at 200 W, 20 kHz for 30 s three times at 30 s intervals. The cytosolic fraction was obtained by ultracentrifugation of the homogenate at 105 000 g for 60 min at 4 ˚C. The HPLC system (Prominence, Shimadzu, Kyoto, Japan) equipped with a narrow bore column was coupled with an ICP-MS (HPLC-ICP-MS) and used for metal speciation.19 The narrow bore gel filtration column (Shodex Protein KW802.5-2E, 2.0 mm i.d. x 250 mm) was kindly provided by Showa Denko (Tokyo). A 5.0 mL aliquot of cytosol was applied to the column and the column was directly eluted with 100 mM ammonium acetate, pH 7.2, at a flow rate of 40 mL / min. The eluate was introduced into a micro concentric nebulizer (Ari Mist HP Nebulizer, Burgener, Ontario, Canada) connected to the cyclone chamber of the ICP-MS. The distributions of Cu and Zn in the eluate were monitored at m/z 65 and 66, respectively.

Evaluation of Atp7a and Atp7b functions in Cu efflux

MT-WT and MT-KO cells were seeded and treated with Atp7a- or Atp7b-specific or control siRNA according to the same protocol as that mentioned above. The cells transfected with siRNA were either exposed or not exposed to 10 mM copper acetate (Wako) plus 30 mM glutathione (GSH; Wako) for 24 h. Since Cu is transported into cells by Ctr1 as a monovalent ion, the complex of Cu(I) and GSH was used. After exposure to the Cu(I)-GSH complex, the cells were washed to remove Cu in the medium and continuously cultured in medium without Cu addition for another 24 h to evaluate the Cu efflux. Then, the cells were collected and wet-ashed with nitric acid to obtain the samples for

Cu determination. Cu concentration was measured with an ICP-MS as mentioned above.

Statistics

Data are presented as means ± S.D. Statistical analysis involved one-way analysis of variance (ANOVA) followed by the Student's t-test. The levels of significant difference were set at p o 0.05 and 0.01 indicated by asterisks (*) and (**), respectively.

Results and discussion

Effects of CCS silencing on CCS and SOD1 expression and SOD1 activity

The protein expression levels of CCS in MT-WT and MT-KO cells decreased to 0.31 and 0.35, respectively, relative to the control when the cells were treated with CCS-targeting siRNA (Fig. 1A and B). There were no significant differences in cell viability between CCS and control siRNA transfected cells in both cell types (data not shown). Thus, this protocol was used to evaluate the effects of CCS knockdown in the following experiments. As CCS plays a role in the delivery of Cu to SOD1, the expression and activity of SOD1 were evaluated in CCS-silenced MT-WT and MT-KO cells. There were no significant differences in the SOD1 expression between CCS and control siRNA transfected cells of both cell types (Fig. 1C and D). SOD1 activity was also not affected by the transfection of CCS-targeting siRNA in both MT-WT and MT-KO cells (Fig. 1E and F). Consequently, although CCS protein expression was actually suppressed by CCS-targeting siRNA, the protein expression and activity of SOD1 were not altered in MT-WT and MT-KO cells.

Cu concentration and distribution in CCS-silenced MT-WT and MT-KO cells

Cu concentrations in MT-WT and MT-KO cells were not changed by the transfection of CCS-targeting siRNA (Fig. 2A and B). Although there were no significant differences in the Cu concentration between MT-WT and MT-KO cells, MT-KO cells tended to have lower concentrations of Cu than MT-WT cells. This observation coincided with previous results.12,15 To evaluate Cu distribution in the cytosolic fraction more precisely, the speciation of Cu by HPLC-ICP-MS was performed. Two major Cu peaks were detected in the chromatogram of MT-WT and MT-KO cells (Fig. 2C and D). The former peak contained not only Cu but also Zn, and its chromatographic behavior was identical to our previous report,20 suggesting that it was assignable to SOD1. The amount of Cu bound to this peak was not changed by the CCS knockdown (Fig. 2C and D). The latter peak containing Cu was assignable to MT isoforms because its chromatographic behavior was identical to that previously reported.20 In the elution profile of MT-KO cells, Cu was eluted at the retention time corresponding to MT (Fig. 2D). The peak containing Cu was also detected in the chromatogram of the liver supernatant of MT-KO mice (data not shown). An unidentified peak containing Cu remains in the chromatogram of the cytoplasmic fraction of MT-KO cells and perhaps also MT-WT cells. None of the peaks in the elution profiles could be assigned to CCS because the amount of CCS protein was estimated to be one-twelfth of that of SOD1 protein.21 Thus, the estimated amount of CCS might be below the detection limit for Cu of the HPLC-ICP-MS under the current conditions. Consequently, the speciation study showed that Cu was constantly distributed in SOD1 in CCS-knockdown cells. No apparent changes in Zn distribution were noted between control and CCS-targeting siRNA treated cells (Fig. 2E and F). Copper chaperones are required for the

proper intracellular delivery of Cu to specific target proteins.22 Indeed, it was shown that CCS is necessary and sufficient for the incorporation of Cu into SOD1 in the presence of a physiological level of Cu in eukaryotes.23–25 It was also demonstrated that the marked reduction of SOD1 activity in tissues of CCS-KO mice (CCS-/-) is caused by the impaired Cu incorporation into SOD1.5 In the literature, SOD1 activity of CCS-/- was maintained at 10–20% in all tissues except the liver, and 30% in the liver of CCS-/-mice in spite of the lack of CCS. Protein blotting analysis showed that SOD1 protein levels in the tissues of CCS-/- were comparable to those of CCS+/- littermates. Hence, SOD1 protein properly received Cu from Cu proteins than CCS. It was speculated that other Cu chaperones or MT compensated the role of CCS. In particular, Atox1 and CCS have a common motif, i.e., MXCXXC, in the Cu-binding domain,26,27 and the expression of other Cu chaperones was actually increased by the CCS knockdown. However, the handing over of Cu to SOD1 requires the formation of a heterodimer between SOD1 and CCS, and the heterodimer is formed on a specific domain of CCS, the so-called domain 3.28 Thus, the formation of the heterodimers of SOD with other Cu chaperones seems to be not feasible. On the other hand, it was demonstrated that MT binding Cu (Cu-MT) was able to deliver Cu to apo-SOD1 in experiments that used both purified proteins.14 However, as SOD1 activity was not affected by the CCS knockdown in MT-KO cells, MT could not play a crucial role in place of CCS in live cells. Contrary to the animal experiments, no apparent decreases in the expression and activity of SOD1 were observed in CCS-knockdown cells (Fig. 1), and SOD1 protein expression was not completely abolished by CCS-targeting siRNA. This suggests that the remaining amount of CCS (31–35% of control) could be sufficient to deliver Cu to SOD1. In other words, the amount of CCS expressed may exceed that required for supplementation of Cu to SOD1 even under physiological conditions. The supplementation of Cu to SOD1 under the Cu-deficient condition was reported in blotchy mutant mice.20 In the literature, blotchy mutant mice having a spontaneous mutation in the Atp7a gene showed systemic Cu deficiency and died within 3 weeks of age. Although the kidney of blotchy mice showed severe Cu deficiency, Cu supplementation to SOD1 was not affected. As another clinical feature, blotchy mice showed hypopigmentation due to a defect in tyrosinase activity, a cuproenzyme. This indicates that Cu supplementation to SOD1 may be predominant compared to other cuproenzymes, at least tyrosinase, and the predominant Cu supplementation to SOD1 may be due to the abundant expression of its specific chaperone, CCS. In addition, it can be suggested that redundant CCS acts as a Cu chaperone and plays other roles in the cells as well. Indeed, it was reported that CCS increased the expression of the reduced form of SOD1 due to its anti-oxidative property.28

Determination of mRNA expression of Cu-related genes in CCS-silenced MT-WT and MT-KO cells

The mRNA expression of CCS in MT-WT and MT-KO cells transfected with CCS-targeting siRNA

decreased to 0.19 and 0.17, respectively, relative to cells transfected with control siRNA (Fig. 3A and 4A). CCS knockdown significantly decreased Ctr1 mRNA expression compared to the control in MT-WT cells (Fig. 3B), while Ctr1 mRNA expression in MT-KO cells showed a tendency to decrease with the transfection of CCStargeting siRNA, similar to the case of MT-WT cells (Fig. 4B). On the other hand, CCS knockdown significantly increased the mRNA expression of Atox1 and Cox17 to 1.25 and 1.23 times higher than those of the control in MT-WT cells, respectively (Fig. 3C and D), and that in MT-KO cells showed the same tendency as well (Fig. 4C and D). Moreover, CCS knockdown significantly increased Atp7a mRNA expression to 3.00 and 2.50 times higher than those of control in MT-WT and MT-KO cells, respectively (Fig. 3E and 4E), and Atp7b mRNA expression showed the same tendency as Atp7a mRNA expression (Fig. 3F and 4F). Meanwhile, SOD1 mRNA expression was not affected by the transfection of CCS-targeting siRNA in both cell types (Fig. 3G and 4G). This was coincident with the protein expression and activity of SOD1 (Fig. 1C, D, E, and F). The mRNA expression of MT-I and MT-II in MT-WT cells was increased to 1.80 and 2.70, respectively, by the transfection of CCS-targeting siRNA (Fig. 3H and I). Such observations as the decrease in Ctr1 mRNA expression and the increase in Atp7a, Cox17, and MT expression could reflect the response to the excess Cu of CCS-knockdown MT-WT and MT-KO cells. Ctr1 mRNA expression was decreased in CCS-silenced MT-WT and MT-KO cells. Ctr1 functions in the high-affinity uptake of Cu on the plasma membrane of various mammalian cells,29–31 and thus, the decrease in Ctr1 mRNA expression is an indication that the cells responded to the excess Cu. On the other hand, the expression of Atp7a and 7b was increased by the CCS knockdown. These Cu-transporting ATPases can act as a Cu-efflux pump and thus, it is straightforward to assume that the increases in Atp7a and Atp7b expression are also a response to the excess Cu. However, Cu concentration in the cells and its distribution in the cytoplasmic fraction were not altered by CCS silencing. Namely, it could be explained that increased Atp7a and/or Atp7b contribute to maintain the intracellular Cu level in CCS-silenced cells. Hence, to evaluate the responsibilities of Atp7a and Atp7b, either ATPases were silenced, and the Cu concentration in MT-WT and MT-KO bearing Atp7a or Atp7b knockdown.

Contribution of Atp7a and Atp7b to the Cu efflux in MT-WT and MT-KO cells

The mRNA expression levels of Atp7a and Atp7b in MT-WT cells introduced with the specific siRNAs were significantly decreased to 0.20 and 0.23 of those of control siRNA-introduced cells, respectively (Fig. 5A and B). On the other hand, the mRNA expression levels of Atp7a and Atp7b in MT-KO cells were also significantly decreased to 0.28 and 0.24, respectively (Fig. 5C and D). These indicated that both Atp7a and Atp7b were expressed in MT-WT and MT-KO cells, and siRNAs used in this study were able to actually reduce the expressions of Atp7a and Atp7b in both cell lines. The Cu concentration in MT-WT cells were significantly increased by the introduction of siRNA

targeting Atp7a, suggesting that the Cu efflux in MT cells were inhibited by silencing Atp7a (Fig. 6A, closed column). There were no significant effects of Apt7b silencing on the Cu concentration in MT-WT cells (Fig. 6A, hatched column). Although MT-KO cells accumulated less Cu than MT-WT as reported previously,15 the results obtained by silencing either ATPase in MT-KO showed the same tendency as those in MT-WT (Fig. 6B). Namely, that the Atp7a knockdown induced Cu accumulation despite Atp7b knockdown not having a significant affect. These results suggest that Atp7a mainly contributes to the Cu efflux in these cell lines, fibroblasts, being independent of the existence of MT. Indeed, this observation was coincident with the gene responses caused by the CCS knockdown (Fig. 3 and 4). The CCS knockdown significantly and specifically induced the Atp7a expression but not Atp7b (Fig. 3E, F, 4E and F) in addition to the reduction of Ctr1 expression. Atp7a substantially responded to the Cu efflux rather than Atp7b (Fig. 6), thus, the maintenance of the intracellular Cu levels in the cells (Fig. 2) would be attributed to these gene responses.

Conclusions

SOD1 activity was maintained in cells even when the amount of CCS was reduced to 0.31–0.35 of the control level by the specific targeting of siRNA. This suggests that the amount of CCS expressed may exceed that required to supply Cu to SOD1. The CCS knockdown caused Cu repletion in the cells, and the Cu-regulating genes, i.e., Ctr1 and Atp7a responded to the Cu repletion resulting in the maintenance of the intracellular Cu level. Our results demonstrate a novel and quantitative relationship between CCS and SOD1, and quantitative contribution of Atp7a and Atp7b in Cu homeostasis.

Acknowledgements

The authors would also like to acknowledge Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Nos. 09J04232 for TM and 19390033 for YO), and the financial support from Agilent Technologies Foundation, USA. The authors wish to thank Showa Denko for providing the narrow bore column and the HPLC system.

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Figures

Fig. 1 Effects of CCS knockdown on CCS and SOD1 protein expression and SOD1 activity in MT-WT and MT-KO cells. CCS (A and B) and SOD1 (C and D) protein expression in MT-WT (A and C) and MT-KO (B and D) cells was determined by Western blotting. SOD1 activity in MT-WT (E) and MT-KO (F) cells was determined by nitroblue tetrazolium staining after native PAGE. SOD activity was detected as a decolorized band on the gel.

Fig. 2 Intracellular Cu concentration and distributions of Cu and Zn in MT-WT and MT-KO cells treated with CCS or control siRNA. Cu concentrations in MT-WT (A) and MT-KO (B) cells were determined with an ICP-MS equipped with a micro flow nebulizer at m/z 65. Data are expressed as means ± SD of three independent determinations. The distributions of Cu (C and D) and Zn (E and F) in MT-WT (C and E) and MT-KO (D and F) cells were determined with the narrow bore HPLC-ICP-MS. The column was eluted with 50mMammonium acetate, pH 7.0, at a flow rate of 40 mL min 1. Cu and Zn in the eluate were monitored at m/z 65 and 66, respectively.

Fig. 3 mRNA expression of Cu-regulating genes in MT-WT cells treated with CCS-targeting or control siRNA. MT-WT cells were treated with CCS-targeting or control siRNA at 100 nM for 24 h. After the siRNA transfection, total RNA was isolated from the cells. The mRNA expression of Cu-regulating genes, such as CCS (A), Ctr1 (B), Atox1 (C), Cox17 (D), Atp7a (E), Atp7b (F), SOD1 (G), MT-I (H), and MT-II (I) was quantified by real-time PCR analysis and normalized to b-actin levels. Data are expressed as means S.D. of three independent determinations.

Fig. 4 mRNA expression of Cu-regulating genes in MT-KO cells treated with CCS-targeting or control siRNA. MT-KO cells were treated with CCS-targeting or control siRNA at 100 nM for 24 h. After the siRNA transfection, total RNA was isolated from the cells. The mRNA expression of Cu-regulating genes, such as CCS (A), Ctr1 (B), Atox1 (C), Cox17 (D), Atp7a (E), Atp7b (F), and SOD1 (G), was quantified by the real-time PCR analysis and normalized to b-actin levels. Data are expressed as means S.D. of three independent determinations.

Fig. 5 mRNA expression of Cu-transporting ATPase genes in MT-WT and MT-KO cells treated with Atp7a- or Atp7b-targeting or control siRNA. MT-WT and MT-KO cells were treated with Atp7a- or Atp7btargeting or control siRNA at 100 nM for 24 h. After the siRNA transfection, total RNA was isolated from the cells. The mRNA expression of Cu-transporting ATPase genes, such as Atp7a (A and C) and Atp7b (B and D) in MT-WT (A and B) and MT-KO (C and D), was quantified by the real-time PCR analysis and normalized to b-actin levels. Data are expressed as means ± S.D. of three

independent determinations.

Fig. 6 Intracellular Cu concentration in MT-WT and MT-KO cells treated with Atp7a- or Atp7b-targeting or control siRNA. MT-WT and MT-KO cells treated with Atp7a- or Atp7b-targeting or control siRNA were exposed 10 mM Cu(I)-GSH complex for 24 h. Then, the Cu(I)-GSH complexwas removed from the medium and the cells were cultured for additional 24 h. Cu concentrations in MT-WT (A) and MT-KO (B) cells were determined with an ICP-MS equipped with a micro flow nebulizer at m/z 65. Data are expressed as means ± SD of three independent determinations.

Fig. 1

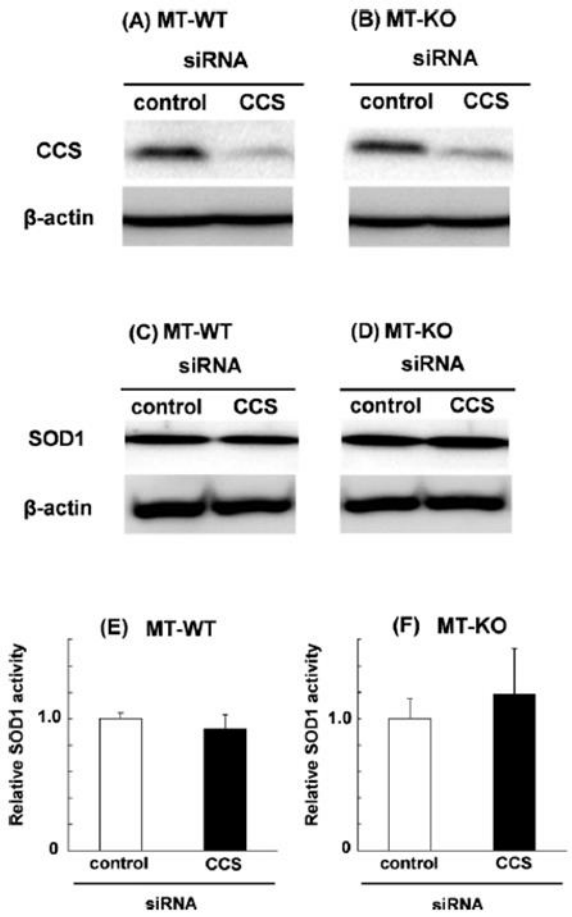

(A) MT-WT
siRNA
control
CCS
CCS
β-actin
(B) MT-KO
siRNA
control
CCS
(C) MT-WT
siRNA
control
CCS
SOD1
β-actin
(D) MT-KO
siRNA
control
CCS
(E) MT-WT
Relative SOD1 activity
1.0
0
control
CCS
siRNA
(F) MT-KO
Relative SOD1 activity
1.0
0
control
CCS
siRNA

Fig. 2

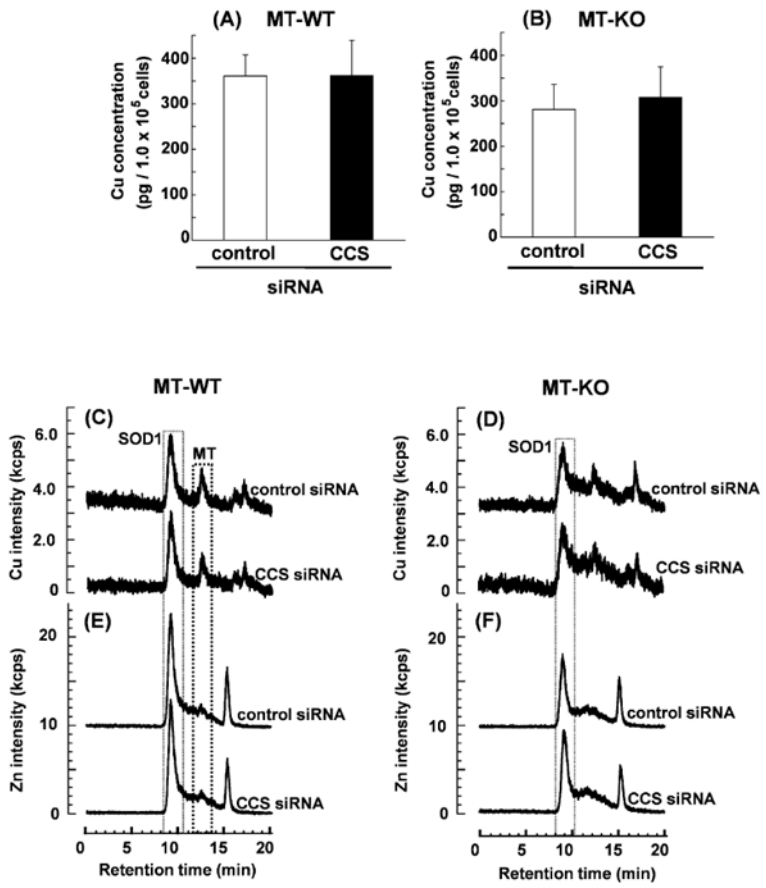

(A) MT-WT
(B) MT-KO
Cu concentration (pg / 1.0 x 10^5 cells)
400
300
200
100
0
control
CCS
siRNA
MT-WT
MT-KO
(C)
(D)
(E)
(F)
SOD1
MT
control siRNA
CCS siRNA
Cu intensity (kcps)
Zn intensity (kcps)
6.0
4.0
2.0
20
10
0
5
15
Retention time (min)

Fig. 3

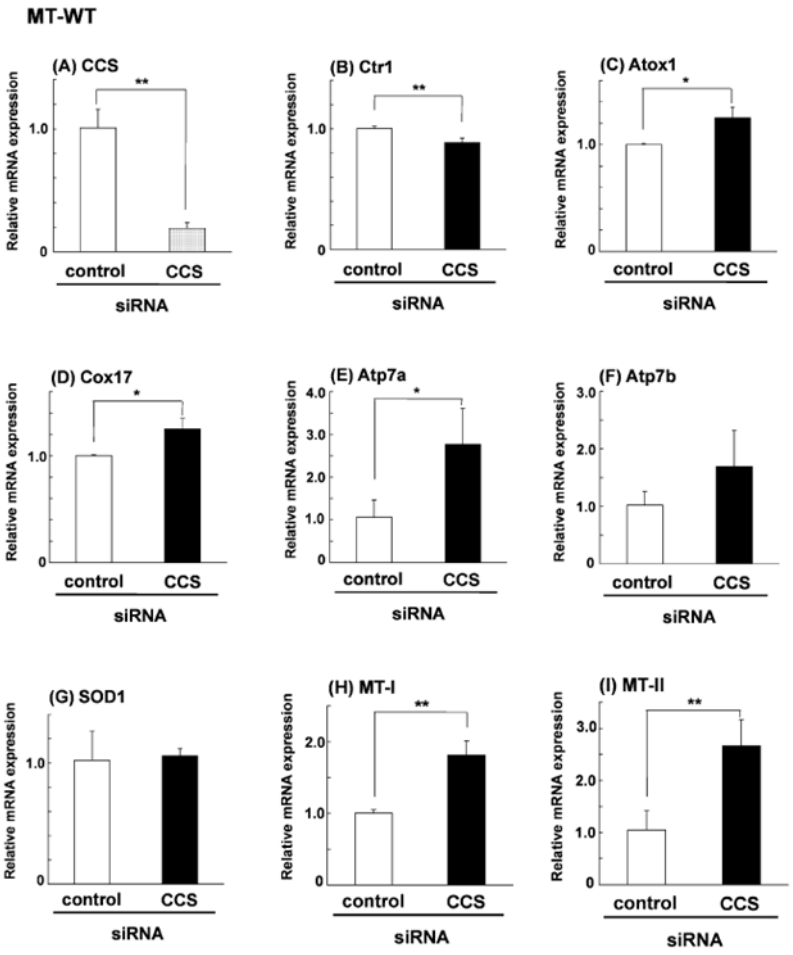

MT-WT
(A) CCS
(B) Ctr1
(C) Atox1
(D) Cox17
(E) Atp7a
(F) Atp7b
(G) SOD1
(H) MT-I
(I) MT-II
Relative mRNA expression
control
CCS
siRNA

Fig.4

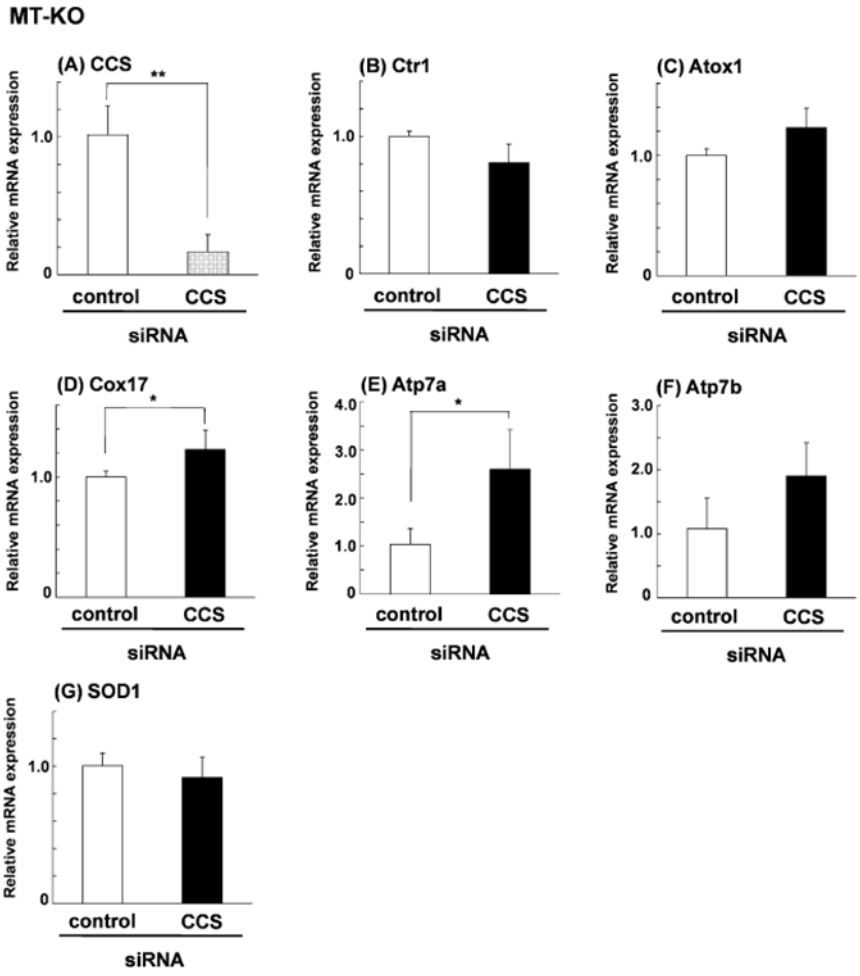

MT-KO
(A) CCS
**
(B) Ctr1
(C) Atox1
(D) Cox17
*
(E) Atp7a
*
(F) Atp7b
(G) SOD1
Relative mRNA expression
1.0
0
2.0
3.0
4.0
control
CCS
siRNA

Fig. 5

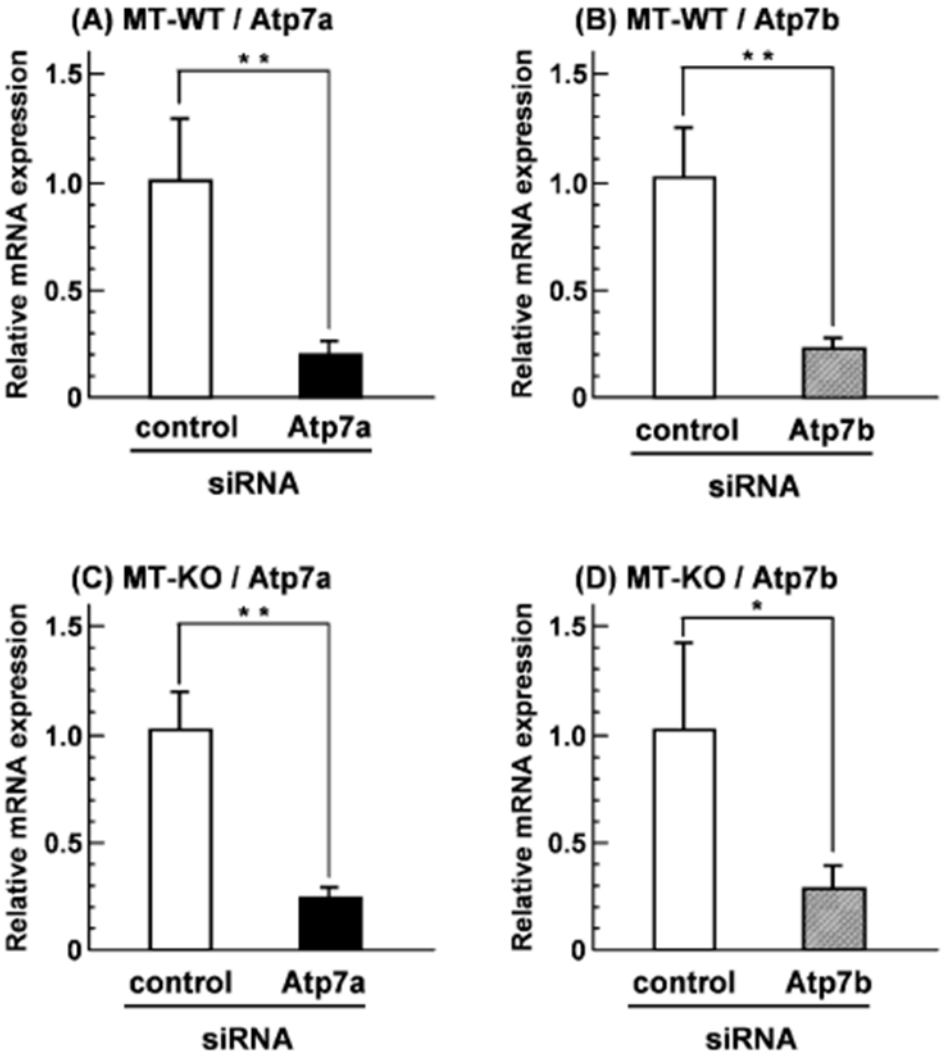
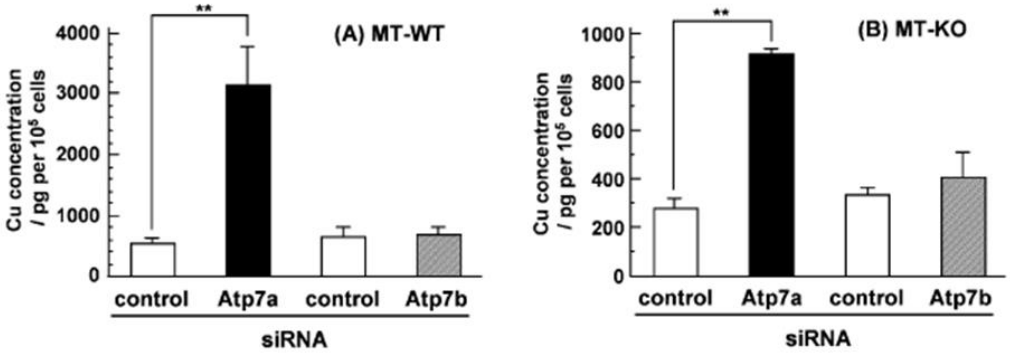

(A) MT-WT / Atp7a
Relative mRNA expression
1.5
1.0
0.5
0
* *
control
Atp7a
siRNA
(B) MT-WT / Atp7b
Relative mRNA expression
1.5
1.0
0.5
0
* *
control
Atp7b
siRNA
(C) MT-KO / Atp7a
Relative mRNA expression
1.5
1.0
0.5
0
* *
control
Atp7a
siRNA
(D) MT-KO / Atp7b
Relative mRNA expression
1.5
1.0
0.5
0
*
control
Atp7b
siRNA

Fig.6


(A) MT-WT
(B) MT-KO
Cu concentration / pg per 10^5 cells
**
4000
3000
2000
1000
0
1000
800
600
400
200
0
control
Atp7a
control
Atp7b
siRNA