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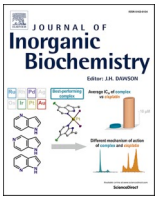

Memo1 reduces copper-mediated reactive oxygen species in breast cancer cells

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ABSTRACT

The mediator of ERBB2-driven cell motility protein 1, Memo1, plays important roles in cancer signaling pathways. We recently reported Memo1 to coordinate reduced copper ions and protect them from reactive oxygen species (ROS) generation in vitro. We here assess if this Memo1 activity is at play in breast cancer cells. Copper additions to MDA-MB-231 cells promoted cell death, and this toxicity was exaggerated when Memo1 expression was reduced by silencing RNA. Using three different commercial ROS probes, we revealed that copper additions increased intracellular ROS levels, and these were further elevated when Memo1 expression was silenced. We propose that, in addition to other functions, Memo1 protects cancer cells from unwanted copper-mediated redox reactions. This may be a required safety mechanism in cancer cells as they have a high demand for copper.

1. Introduction

Living organisms depend on copper (Cu) ions for survival [1]. Cu is used as a redox-active cofactor in many important enzymes that, for example, facilitate respiration, pigmentation, and neuropeptide synthesis. Because of its cofactor role in many fundamental enzymes, Cu is linked to many hallmarks of cancer, e.g., proliferative immortality, angiogenesis, and metastasis [2–6]. In accord with high demand, both cancer tissue and cancer patients' blood contain elevated levels of Cu [7]. If uncontrolled, the redox activity of Cu can promote the formation of reactive oxygen species (ROS) that damage proteins, DNA and membranes [8]. Therefore, cells have dedicated Cu transport systems that facilitate the timely and safe delivery of Cu to Cu-dependent enzymes [9–12]. Even if ROS, including hydrogen peroxide (H_2O_2), superoxide, hydroxyl radical, and singlet oxygen [13], can harm cells, they are also used for signaling. Not only Cu can generate ROS, additional pathways for ROS generation also exist [14]. The increased metabolic activity of cancer cells results in higher ROS levels than in normal cells [15]. Still, like with too much Cu, too much or uncontrolled formation of ROS can kill cancer cells [16,17]. Maintaining high, but not too high, levels of Cu and ROS is crucial for survival of cancer cells.

Mediator of ERBB2-driven Cell Motility 1 protein (Memo1) is a protein involved in many cancer-related signaling pathways. Initially, it was suggested to promote growth of lamellipodia and cancer cell migration through its interaction with the ERBB2 receptor [18]. Overexpression of Memo1 increased cell migration in a breast cancer wound closure assay and high levels of Memo1 was found to correlate with worse survival of breast cancer patients [19]. Over the years, many functionalities have been attributed to Memo1, such as in embryonic development and adult organ sustainability [20]. Because of the cancer-promoting functions, Memo1 has been suggested as a cancer drug target.

The reported high-resolution crystal structure of Memo1 showed an overall fold similar to that of non-heme iron dioxygenases except that the metal-binding site included a Cys instead of a Glu residue (the other putative metal-site resides, three His and one Asp, were the same) [21]. Early efforts to bind various metal ions (e.g., iron and zinc) to Memo1 failed; instead, it was proposed that the metal-site region in Memo1 acted as a binding site of the ERBB2 receptor peptide [22].

In 2014, a study suggested that Memo1 could bind Cu(II) and catalyze Cu-dependent ROS production in cancer cells [19]. However, when we purified Memo1 protein and tested this possibility at controlled conditions in vitro, we found no Cu(II) binding. In contrast to the earlier study, we discovered that Memo1 coordinates two reduced (Cu(I)) Cu ions per protein with high affinity. When Cu was bound to Memo1, it was protected from ROS-generating redox reactions in vitro [23]. To extend our in vitro findings to living cancer cells, we here assessed ROS levels in MDA-MB-231 breast cancer cells as a function of Memo1 expression and addition of excess copper. The results presented show that our previous in vitro results [23] hold true also in a breast cancer cell line.

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2. Results and discussion

We first tested the effect of added external Cu on cell viability using the breast cancer cell line MDA-MB-231 (which we used in previous work [23]) and Cu levels up to 800 μM. We note that MDA-MB-231 is a triple negative cell line and future studies should include estrogen-receptor positive breast cancer cells. Using the common cell viability assay based on MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] that probes active metabolism [24], we found that incubation of cells with increasing levels of external copper (18 h incubation), decrease cell viability (Fig. 1A). The addition of copper did not signficantly affect the expression level of Memo1 in the cells (Fig. 1B).

We next tested the effect of added external copper on cancer cell viability when the amount of Memo1 protein was reduced using siRNA. Memo1 silencing reduced the protein level down to about 20–30% of the normal Memo1 level in cells (Fig. 1C). Cell viability was significantly more decreased for cells with Memo1 silenced, as compared to those with Memo1 present, at all different Cu additions (Fig. 1A). It is important to point out that although Cu is added in the Cu(II) form, Cu uptake in cells takes place in the Cu(I) form [25,26]. Moreover, even though the added amounts of Cu is very high, only a fraction of this Cu is internalized [1].

We then measured ROS levels in the cells using three different commercial ROS probes at four conditions: 0 and 350 μM Cu added conditions, each with and without Memo1 levels reduced via siRNA. We selected 350 μM Cu as our high-Cu condition as, at that added Cu concentration (18 h incubation), there was only modest effect on cell viability. For each experiment, we performed two biological replicas with four technical repeats in each. Silencing efficiency of Memo1 expression was tested in every new batch of cells, and in all biological replicas shown here, Memo1 expression was reduced between 70 and 80 % at the Memo1 siRNA conditions (examples, Fig. 1C).

Total H_2O_2 levels were probed using the ROS-Glo™ assay. H_2O_2 is convenient to assay because it has the longest half-life of all ROS in cultured cells and other ROS may be converted to H_2O_2 [27,28]. No significant difference in H_2O_2 levels was found between Memo1 knockdown and control cells in absence of Cu addition, but with 350 μM Cu added, the H_2O_2 level increased by about 50% in Memo1 knockdown cells (Fig. 2).

The oxidative state, using CellROX™ Green, and mitochondrial superoxide level, using MitoSOX™ Red, were also measured at these conditions. CellROX™ Green is a dye that upon oxidation binds to DNA in the nucleus and mitochondria and gives green fluorescence. MitoSOX™ Red is a dye targeted to mitochondria that is rapidly oxidized by superoxide, but not by other ROS or reactive nitrogen species. The oxidized product is highly fluorescent and thus reports on superoxide production in mitochondria. Flow cytometry experiments (Fig. 3 AD) with these probes show that Cu addition increases ROS in the cells; this

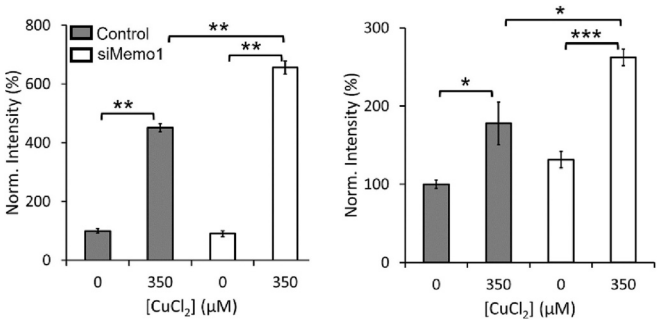

Fig. 2. H_2O_2 levels as a function of Cu and Memo1. Normalized luminescence intensity of cells with (siMemo1) and without (control) Memo1 silencing at 18 h incubation with no and 350 μM Cu in the ROS-Glo H_2O_2 assay. Two biological replicas are shown. Error bars represent standard error of means (SEM) ($n = 4$ per condition). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

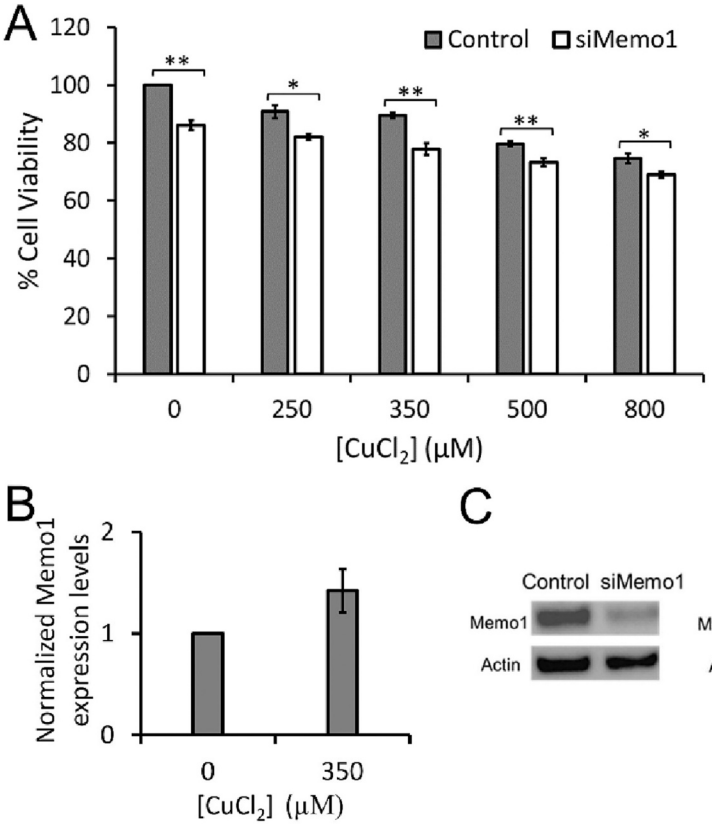

Fig. 1. A. Cancer cell viability as a function of Cu and Memo1. MDA-MB-231 cells were incubated with 0, 250, 350, 500 and 800 μM $CuCl_2$ for 18 h, followed by MTT assay for cell viability analysis. Control means cells with Memo1 present, adding a control siRNA, and siMemo1 means cells with siRNA added targeting Memo1 (Memo1 knockdown) and, thus, the latter cells have reduced Memo1 protein level (see C). Error bars represent standard error of means (SEM) ($n = 4$ per condition), *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. B. Memo1 expression level (normalized) in cells treated with 0 and 350 μM Cu for 18 h. Two biological replicas. Difference was not signficiant. C. Examples of Western blotting of Memo1 silencing efficiency in cells. Expression was reduced 70–80% in all biological replicas used in experiments. Left: cells used in one replica of ROS Glo H_2O_2 assay (reported in Fig. 2) (here, Memo1 expression was reduced by 70%). Right: cells used in one replica of CellROX Green and MitoSOX Red experiments (reported in Fig. 3) (here, Memo1 expression was reduced by 75%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

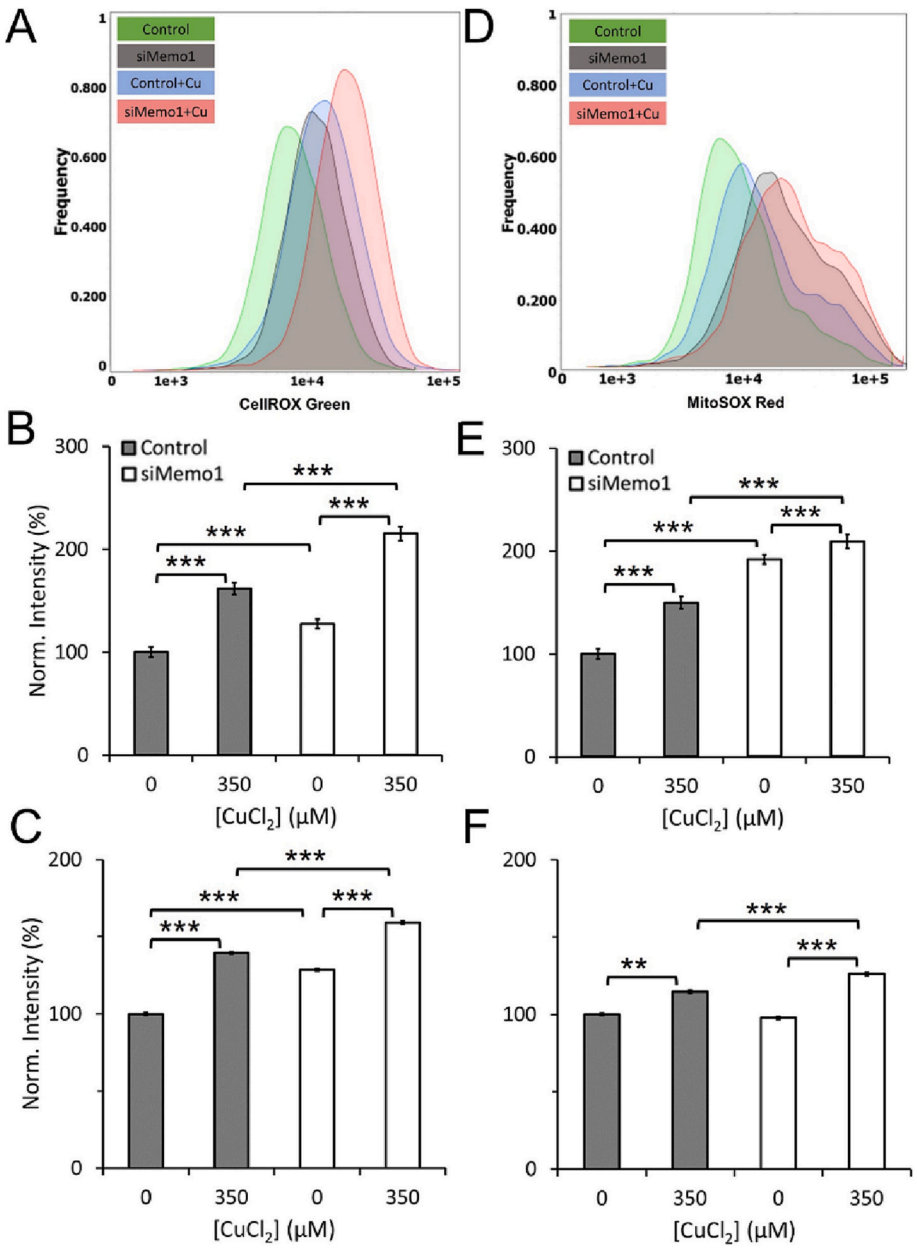

Fig. 3. ROS levels as a function of Cu and Memo1. (AD) Examples of flow cytometry data and (BCEF) analysis of ROS production in cells (detected as dye fluorescence) with and without Memo1 silencing incubated with and without 350 μM Cu for 18 h on 12-well conventional plates. Normalized fluorescent intensities of CellROX Green (BC) and MitoSOX Red (EF). Two biological replicas are shown for each probe. Error bars represent standard error of means (SEM) ($n = 4$ per condition). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effect is further exaggerated by Memo1 knockdown for both ROS probes (Fig. 3 BCEF). At both 0 and 350 μM Cu conditions, removal of Memo1 protein results in increased ROS. For CellROX Green, the difference between cells with and without Memo1 is significant for both 0 and 350 μM Cu conditions in both biological replicas (Fig. 3 BC). For MitoSOX, the increase in ROS for cells without Memo1 (as compared to with Memo1) is significant for 350 μM Cu in both biological replicas, but at the 0 μM Cu condition, only one of the biological replicas shows a significant increase without Memo1 (Fig. 3 EF).

Taken together, when breast cancer cells are challenged by excess Cu, more ROS is detected in the cells, in accord with expected Cu-mediated ROS generation. We here show that the presence of Memo1 in these breast cancer cells reduces ROS levels as compared to ROS levels for cells with knockdown of Memo1 expression. All three ROS probes showed this effect of Memo1 when the cells were challenged with excess Cu (350 μM Cu, 18 h incubation). The results are not as clear when analyzing the results for no copper added. Although there is a tendency for increased ROS levels when Memo1 is silenced also at 0 μM Cu added, the observations at 0 μM Cu are only significant for the CellROX Green probe. We note that condition with added external Cu (ROS difference significant for all probes) may be the best mimic of cancer cells in a tumor, as cancer tissue in vivo is found to have elevated levels of Cu [7].

The in-cell results obtained here agree with what we recently reported for purified Memo1 protein in vitro. We found, in contrast to previous work [19], that Memo1 coordinates two reduced Cu ions (Cu (I)) and by doing so, the Cu ions are shielded from redox cycling (and thus generation of ROS) in vitro [23]. We explain the difference with the McDonald study [19] by they using excess Cu in the in vitro studies that, in our hands, results in Memo1 precipitation [23]. Moreover, the McDonald study used a single ROS probe for cell work (CellROX Red; which is very similar to the CellROX Green used here) and specifically analyzed cell protrusion regions [19]. It is possible that ROS levels vary locally within the cells. We measured total ROS in the cells, although the three commercial probes we used have different specificities and limitations. Further work with genetically engineered ROS sensors is desired in combination with subcellular localization studies. In agreement with our current findings, work in *Caenorhabditis elegans* and mice have shown that loss of Memo1 is associated with an increase in ROS in the

organism [29].

In conclusion, we speculate that since cancer cells have increased Cu levels, erroneous free Cu ions promoting toxic ROS may be a larger threat than in normal cells (with lower Cu levels) [7]. To protect against this, Memo1 may act as a Cu chelator to protect cells from uncontrolled ROS formation (Fig. 4). Since the affinity of Cu is higher for the cytoplasmic Cu chaperone Atox1 than it is for Memo1 [23], it is possible that Memo1 channels Cu to Atox1 for Atox1-mediated delivery of Cu into the secretory pathway via which Cu can be exported out of the cell.

3. Materials and methods

Cell lines, siRNAs, and reagents. The MDA-MB-231 human breast cancer cell line was obtained from American Type Culture Collection (LGC standards). The cells were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (Gibco), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen). The passage number was kept below 20, and the cells were tested for mycoplasma periodically. Control (siCtrl) and Memo1 silenced (siMemo1) MDA-MB-231 cells were generated by transient transfection with AllStars negative control siRNA or a pool of four different siRNA targeting Memo1 (Qiagen), using Lipofectamine RNAiMAX transfection reagent (Invitrogen). Memo1 silencing efficiency was always tested by Western blotting with actin as a control. $CuCl_2$ and MTT formazan was purchased from Sigma-Aldrich. CellROX™ Green (5 mM solution) and MitoSOX™ Red powder were purchased from ThermoFisher Scientific. ROS-Glo™ H_2O_2 kit was purchased from Promega Biotech AB.

Western blotting. Proteins were extracted by lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton and 100 U/ml protease inhibitor cocktail (Roche). Total protein concentration was measured by the colorimetric bicinchoninic acid (BCA) protein assay reagent (Alfa Aesar). Equal amount of total protein for each sample was subjected to SDS/PAGE and transferred onto PVDF membranes (Bio-Rad). Membranes were first probed with primary antibodies: mouse anti-Memo1 antibody (sc-517,412, Santa Cruz) and rabbit anti-beta actin antibody (ab8227, Abcam), and subsequently probed with HRP-conjugated goat anti-rabbit secondary antibody (Sigma) and Mouse IgG HRP Linked Whole Ab (GENA931, Sigma-Arldrich). Memo1 and actin protein bands were detected using the enhanced chemiluminescence plus Western blotting detection reagents (GE Healthcare) and ChemiDoc™ touch imaging system (Bio-Rad).

ROS measurements. CellROX™ Green and MitoSOX™ Red were used to detect ROS levels. Control and Memo1 knockdown cells with and without 18 h incubation with 350 μM $CuCl_2$ were seeded on a 12-well plate. The cells were harvested with trypsin-EDTA (Sigma) and CellROX Green reagent was added to a final concentration of 5 μM and incubated for 30 min at 37 °C in the dark. The medium was then removed, and cells were washed three times with PBS. The cells were subjected to flow cytometry using an Amnis® CellStream® Flow Cytometer (Luminex®) (ex/em 485/520 nm). Approximately 10^4 cells were analyzed in each of the samples—four replicates per condition.

Mitochondrial superoxide production was monitored using MitoSOX Red. Before use, MitoSOX Red was dissolved in DMSO to yield a 5 mM stock that was then diluted to 5 μM in warm HBSS (with calcium and magnesium). Control and Memo1 knockdown cells with and without 18 h incubation with 350 μM $CuCl_2$ were treated with MitoSOX red (5 μM) for 10 min at 37 °C in the dark. The cells were washed, and then subjected to flow cytometry using a flow cytometer (ex/em: 386/610 nm). Approximately 6000 cells were analyzed in each of the samples. Four replicates per condition.

For the ROS-Glo™ H_2O_2 assay, 10^5 cells (with and without siMemo1) were seeded in a 96 well plate. After 18 h incubation with and without 350 μM $CuCl_2$, the assay was performed as directed by the manufacturer. The H_2O_2 substrate solution was added, bringing the final volume to 100 μl. The plate was incubated at 37 °C in a 5% CO_2 incubator for 6 h. 100 μl of the ROS-Glo detection solution was then added to each well.

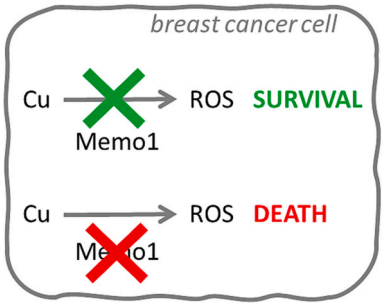

Fig. 4. Proposed role of Memo1 in breast cancer. Memo1 can scavange and redox-silence Cu ions that otherwise would create dangerous ROS. This Memo1 functionality results in decreased cell vulnability to high Cu levels (which are needed for cancer-promoting functions). Thus, Memo1 promotes cancer cell survival via protection against uncontrolled ROS formation by erronoeous free Cu ions.

After an additional 20 min incubation at room temperature, luminescence was recorded using a luminometer (CLARIOstar). Four replicates were performed per condition.

All three ROS probe experiments were repeated twice, starting from scratch, to give two biological replicas of each data set. Statistical analysis of significant differences between conditions was performed using paired *t*-tests.

Cell viability analysis. Control and Memo1 knockdown cells were seeded onto 96-well plates at a density of 10^4 cells per well for 24 h, followed by incubation with or without various concentrations of $CuCl_2$ for 18 h. The culture media was then changed to serum-free media. MTT dissolved in PBS was added to each well and incubated for 4 h. Then the media was removed and DMSO added into each well. The plates were vortexed at a low speed for 10 min to fully dissolve the crystals. Absorbance was measured at 570 nm using a microplate reader (CLARIOstar). Four replicates were performed per condition. Two biological replicas were performed.

Author statement

All authors contributed to this work and approved the submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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