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Research Paper

A Role for The ATP7A Copper Transporter in Tumorigenesis and Cisplatin Resistance

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Abstract

The ATP7A protein is a ubiquitously expressed copper-translocating P-type ATPase that controls cytoplasmic copper concentrations by mediating cellular copper egress. *In vitro* studies have previously demonstrated that ATP7A abundance in various tumor cell lines is correlated with increased resistance to cisplatin, a widely-used chemotherapy agent. However, to date no studies have examined a role for ATP7A in tumor growth or cisplatin sensitivity *in vivo*. In this study, we deleted ATP7A in H-RAS transformed tumorigenic mouse embryonic fibroblasts (MEF^{RAS}7A-). Interestingly, loss of ATP7A was found to markedly suppress tumorigenesis in MEF^{RAS}7A- cells relative to wild type parental cells. This was associated with hyperaccumulation of copper and sensitivity to reactive oxygen species and hypoxia. Tumor grafts lacking ATP7A were markedly more sensitive to cisplatin chemotherapy compared to ATP7A-expressing control tumors. These findings identify ATP7A at the nexus between tumorigenesis and cisplatin resistance pathways, underscoring its potential as a therapeutic target for regulating both tumor growth and the efficacy of cisplatin treatment.

Key words: ATP7A, Copper transporter, Cisplatin resistance, Tumorigenesis

Introduction

Copper is an enzymatic cofactor that in mammals is necessary for cellular respiration, iron homeostasis, melanogenesis, neurotransmitter biosynthesis, and connective tissue formation. The essentiality of copper lies in its ability to cycle between Cu^{1+} and Cu^{2+} oxidation states, which confers upon cuproenzymes the ability to catalyze redox reactions. However, this same redox property also underlies its potential toxicity if copper concentrations surpass the capacity of cellular detoxification mechanisms. The principle regulators of copper homeostasis in virtually all cell types include CTR1, a plasma membrane copper importer, and ATP7A, a copper exporting P-type ATPase that regulates cytoplasmic copper concentrations. The intracellular roles of ATP7A include the delivery of copper to essential cuproenzymes of the secretory pathway, and copper excretion across the plasma membrane [1-3]. Accordingly, loss of ATP7A in cultured cells results in the accumulation of copper in the cytosol, and hypoactivity of various copper-dependent enzymes that require copper to be transported into the secretory pathway [4-7].

Previous studies have shown that copper transporters play key roles in the cellular accumulation of the chemotherapy agent cisplatin [8-12], a front-line treatment against several types of cancer [13]. As with many chemotherapy agents, acquired cisplatin resistance becomes problematic upon repeated exposure [14, 15]. The expression of

CTR1 in cultured cells is inversely proportional to cisplatin accumulation and resistance, suggesting that CTR1 is responsible for cisplatin uptake across the plasma membrane. In contrast, ATP7A abundance is positively correlated with increased resistance to cisplatin [16-18]. Elevated expression of ATP7A is associated with worse outcomes in patients with ovarian or non-small cell lung cancer who are treated with platinum- containing drugs [19-21]. Similar correlations have been made between cisplatin resistance and a closely related copper ATPase, ATP7B [22]. Although the mechanisms by which ATP7A/B contribute to cisplatin resistance are not well understood, several hypotheses have been proposed including direct transport and sequestration of the drug into intracellular vesicles, or chelation through interactions between cisplatin and metal-binding domains in these proteins [23-25]. In addition to playing roles in cisplatin resistance, pathways of copper homeostasis also appear to regulate tumor growth, although the underlying mechanisms are poorly understood. Changes in systemic copper metabolism are known to accompany various types of cancer. Serum copper levels are elevated in cancer patients, which correlate with disease outcome and response to therapies [26, 27]. Copper-chelating drugs such as tetrathiomolybdate or penicillamine are known to have anti-angiogenic and anti-neoplastic activity, indicating that copper is rate limiting for tumor growth [28-31]. Copper chelation has been shown to reduce ATP levels and suppress pancreatic tumors in mice, in part due to restricting the activity of copper-dependent mitochondrial cytochrome c oxidase [32]. Other studies suggest that NFkappaB-dependent tumorigenesis is also impaired by copper chelation [30]. Copper also appears to be rate limiting for mitogenic signaling pathways necessary for tumor growth such as RAS/MAPK signaling, in part due to a direct role for copper ions in regulating MEK1 activity [33]. Studies have also shown that copper-dependent superoxide dismutase is necessary for receptor tyrosine kinase signaling by producing hydrogen peroxide, which blocks downstream inhibitory phosphatases [34]. These studies suggest that cancer cells are vulnerable to disturbances in copper homeostasis, a weakness that might be exploited by novel therapeutic strategies to restrict copper delivery to one or more key enzymes with roles in cancer biology.

The goal of the current study was to investigate the extent to which ATP7A is necessary for tumor growth and cisplatin resistance *in vivo*. Deletion of *ATP7A* in tumorigenic fibroblasts was found to significantly increase cellular accumulation of copper, sensitize these cells to both hypoxia and reactive oxygen species, reduce tumor formation in mice, and increase cisplatin sensitivity *in vitro* and *in vivo*. These data reveal a requirement for ATP7A in both tumor growth and cisplatin resistance, highlighting its potential as a novel target for anti-cancer therapies.

Results and Discussion

Generation of tumorigenic ATP7A knockout MEF cells. An initial goal of this study was to generate a tumorigenic mouse embryonic fibroblast cell line containing a floxed *ATP7A* allele that would enable subsequent *ATP7A* gene deletion using Cre recombinase. Previously, we generated a mouse embryonic fibroblast cell line carrying a floxed *ATP7A* allele (MEF7A fl/Y) that was immortalized by expression of the Simian virus 40 (SV40) large T-antigen [35] (Figure 1A). As transformation usually requires collaboration between at least two oncogenes in rodent cells, we transfected these cells with a plasmid carrying a mutant *RAS* oncogene (H-RAS^{V12}). Several independent clones were isolated and tested for tumorigenicity by subcutaneous injection into immunocompromised mice. A single clone was chosen for further study, hereafter called MEF^{RAS}7A+, which gave rise to palpable tumors at 100% incidence with a latency period of approximately 10 days (Figure S1). To generate isogenic clones in which ATP7A was deleted, the MEF^{RAS}7A+ cells were infected with adenovirus carrying the Cre recombinase gene, and single clones were isolated in which ATP7A expression was absent. One of these clones, MEF^{RAS}7A- was chosen for further study (Figure 1A and 1B). Consistent with the known role of ATP7A in copper efflux, the MEF^{RAS}7A- cells were found to hyperaccumulate copper relative to MEF^{RAS}7A+ cells (Figure 1C). The MEF^{RAS}7A- cells also exhibited lower levels of CCS (Figure 1B), a protein whose abundance is inversely proportional to cytoplasmic copper concentration [35, 36]. No differences were found in the levels of the ATP7B protein, which was weakly detected in both MEF^{RAS}7A+ and MEF^{RAS}7A- cells. A similar level of expression of the CTR1 copper importer was found in both cell lines (Figure 1B). Despite the hyper-accumulation of copper in MEF^{RAS}7A- cells, these cells grew at a similar rate compared to control MEF^{RAS}7A+ cells in regular growth medium (Figure 1D).

ATP7A deletion suppresses tumorigenesis. To investigate the effect of *ATP7A* gene deletion on tumor growth, the MEF^{RAS}7A- and MEF^{RAS}7A+ cells were subcutaneously injected into nude mice, and tumor volume was measured at regular intervals over 5 weeks. Tumors derived from MEF^{RAS}7A- cells were found to be markedly smaller than those from

MEF^{RAS}7A+ cells (Figure 2A-C). This difference in tumor size was observed in multiple independent clones of MEF^{RAS}7A- cells (data not shown). Histological analyses of the tumors did not reveal obvious differences in markers of vascular density (CD31) or proliferation (Ki67) (Figure S2). The findings suggest that loss of ATP7A is tumoristatic in RAS-transformed fibroblasts.

Loss of ATP7A causes sensitivity to ROS and hypoxia. The observation that MEF^{RAS}7A- cells grew at a similar rate as MEF^{RAS}7A+ cells in cell culture media, and yet showed reduced tumor growth in mice, suggests that the growth inhibition mediated by ATP7A deletion is specific to *in vivo* conditions. As the rapid growth of solid tumors is often associated with hypoxia and oxidative stress, we investigated whether MEF^{RAS}7A- cells might be more sensitive to hypoxia and/or hydrogen peroxide compared to MEF^{RAS}7A+ cells using an MTT assay of cell viabiility. Hydrogen peroxide alone, or in the presence of hypoxia, resulted in a significant inhibition of growth in MEF^{RAS}7A- cells relative to MEF^{RAS}7A+ cells (Figure 3). Importantly, this hypersensitivity was prevented by the addition of the membrane permeable copper chelator, tetra-thiomolybdate (Figure 3). Similar results were obtained using a Crystal violet assay of cell proliferation (Figure S3). These findings are consistent with the hypothesis that loss of ATP7A renders MEF^{RAS}7A- cells hyper-sensitive to ROS in a copper dependent manner.

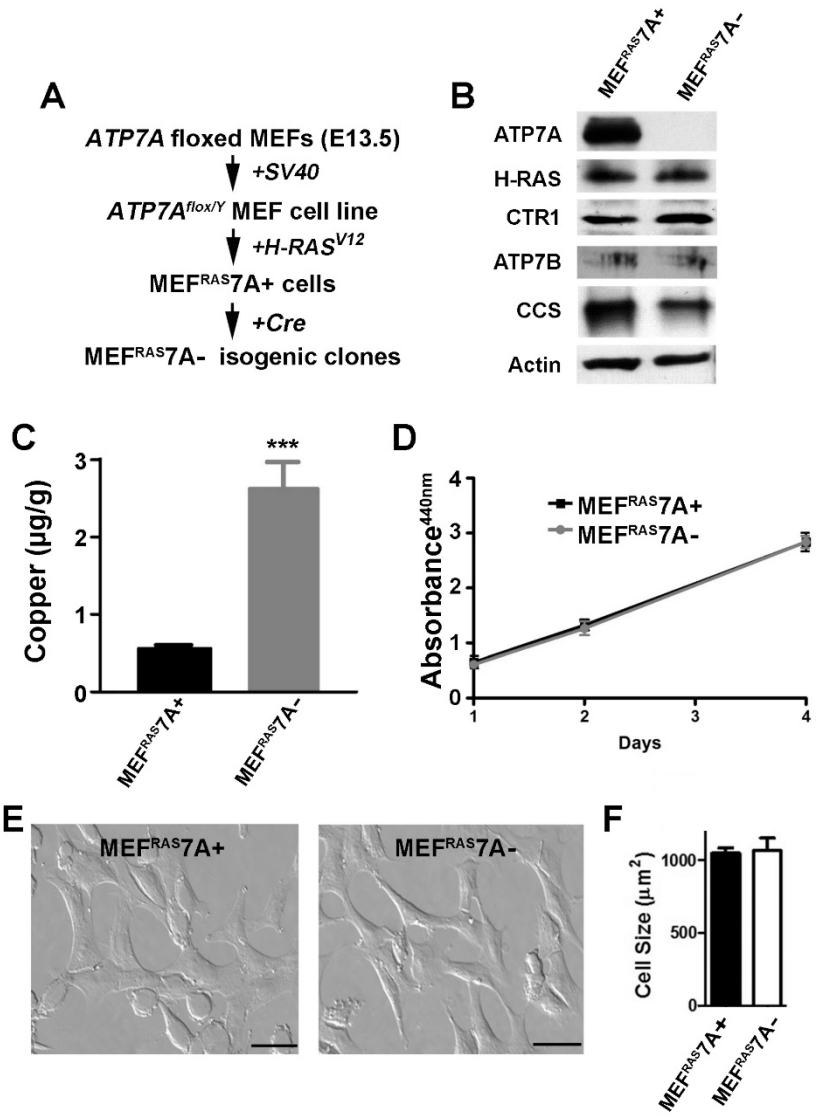

Figure 1. Characterization of MEF^{RAS}7A+ and MEF^{RAS}7A- cells. (A) MEF^{RAS}7A+ cells were generated from a mouse embryonic fibroblast cell line carrying a floxed *Atp7a* allele and transformed by sequential transfection of SV40 large T-antigen and mutant H-RAS oncogene. Isogenic MEF^{RAS}7A- cells were generated by infecting with adenovirus carrying the Cre recombinase. (B) Immunoblot analyses. Cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. (C) Copper concentration determination within cultured MEF^{RAS}7A+ and MEF^{RAS}7A- cells by inductively coupled plasma mass spectrometry (mean ± S.E.M; ***P<0.001; n = 3). (D) Cell proliferation was evaluated using WST-1 colorimetric assay by measuring the absorbance at 440nm (mean ± S.E.M; n = 3). (E) Representative images of MEF^{RAS}7A+ and MEF^{RAS}7A- cells Scale bars: 50µM. (F) Cell size determination of MEF^{RAS}7A+ and MEF^{RAS}7A- cells (mean ± S.E.M; n = 30 cells in three fields).

ATP7A contributes to cisplatin resistance in vitro and in vivo. While previous studies have demonstrated that ATP7A plays a role in cisplatin resistance in cultured cell lines, there have been no studies examining this requirement *in vivo* [16]. In support of previous studies, the MEF^{RAS}7A- cells exhibited reduced survival compared to MEF^{RAS}7A+ cells in cell culture medium containing cisplatin (Figure 4A). To test the *in vivo* requirement for ATP7A in cisplatin resistance, we compared the inhibitory effect of cisplatin chemotherapy on tumor growth of MEF^{RAS}7A- and MEF^{RAS}7A+ cells in mice. Tumor growth was initiated by subcutaneous injection of either MEF^{RAS}7A- or MEF^{RAS}7A+ cells in nude mice. After a 10-day initial growth period, cisplatin (4mg/kg) or saline control was administered via intraperitoneal injection at weekly intervals over a three-week period, and tumors were then harvested at day 40 (**Figure 4B**). Consistent with our earlier observations, in the saline-treated control animals the MEF^{RAS}7A- tumors were markedly smaller than the MEF^{RAS}7A+ tumors (Figure 4C and 4D). Importantly, cisplatin treatment significantly reduced the size of the MEF^{RAS}7A-tumors compared to the

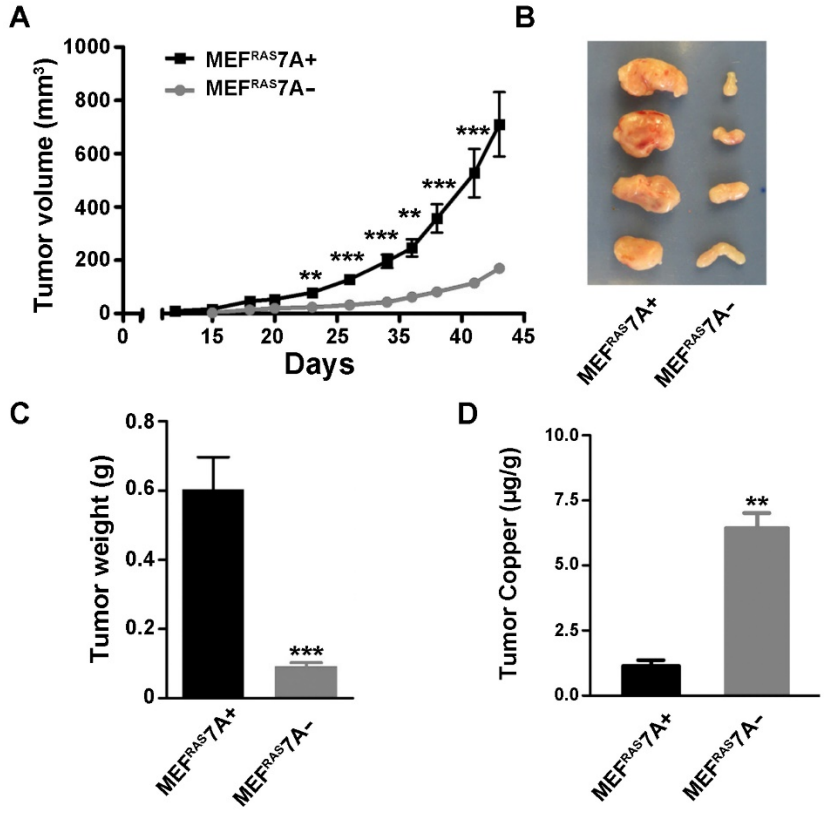

Figure 2. Deletion of *Atp7a* suppresses tumor growth of MEF^{RAS}7A- cells Subcutaneous growth of MEF^{RAS}7A+ and MEF^{RAS}7A- cells in nude mice. Tumor volume (A) and tumor weight (C) are shown. B) Representative MEF^{RAS}7A+ and MEF^{RAS}7A- tumors at 5 weeks after cell implantation (n= 10-12 mice; mean ± S.E.M; ***P*<0.01; ****P*<0.001). (D) Tumor copper concentrations measured by ICP-MS (mean ± S.E.M; ***P*<0.01).

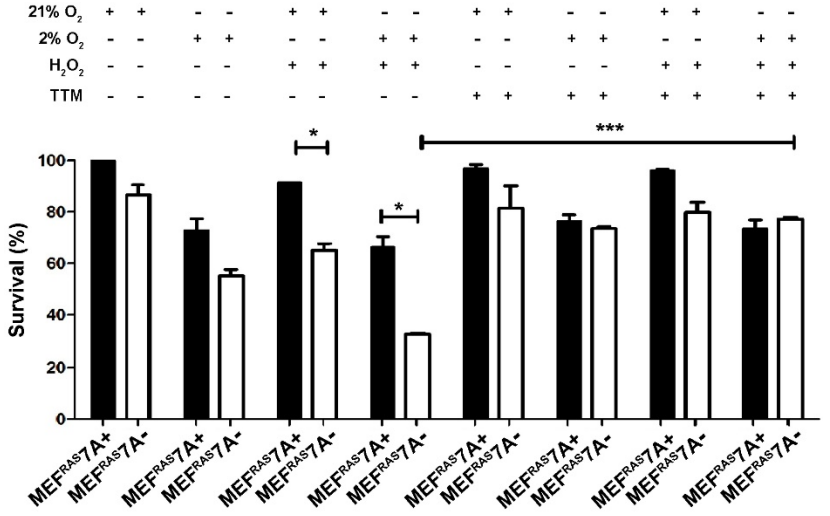

Figure 3. Loss of ATP7A confers copper-dependent hypersensitivity to hydrogen peroxide. MEF^{RAS}7A- and MEF^{RAS}7A+ cells in a 24 well plate were exposed to 40 µM H_2O_2 in either normoxia (21% O_2) or hypoxia (2% O_2) in the presence of absence of the copper chelator tetrathiomolybdate (TTM; 4µM). Cell viability was then determined using the MTT assay after 24 hours of treatment (mean ± S.E.M., **P*<0.05, ***P*<0.01, ****P*<0.001).

saline treated controls, however, there was no significant inhibitory effect of cisplatin on the growth of MEF^{RAS}7A+ tumors (Figure 4C and 4D).

Taken together, the results of this study indicate that deletion of the *ATP7A* gene is rate limiting for tumor growth, and enhances the efficacy of cisplatin-mediated chemotherapy in a RAS- transformed tumor model. Loss of ATP7A protein attenuated the growth of MEF^{RAS}7A- tumors in mice compared to wild type cells, but had little impact on their growth in cell culture. The tumoristatic effects of copper hyperaccumulation have been demonstrated in previous studies involving deletion of a related copper exporter, ATP7B [37, 38]. However, the tumoristatic effect of ATP7A deletion in our study was not solely attributable to copper hyperaccumulation *per se*, because the magnitude of the increased copper accumulation in MEF^{RAS}7A- cells *in vitro* was similar to that in MEF^{RAS}7A- tumors. These findings suggest that the loss of ATP7A was deleterious specifically within the tumor microenvironment. It is well documented that cancer cells produce increased levels of ROS relative to normal cells [39-42], and that copper ions are known to potentiate the toxicity of ROS, in part, by catalyzing Fenton-like chemistry [43]. Our finding that cultured MEF^{RAS}7A- cells were more sensitive to hypoxia and / or hydrogen peroxide relative to wild type cells offers a possible model by which for the tumoristatic effect of ATP7A deletion by augmenting the deleterious effects of ROS generated within the hypoxic environment of the tumor. This model does not exclude the possibility that loss of ATP7A may perturb other pathways important for tumor growth. Indeed, because ATP7A functions to transport copper to nascent polypeptides within the secretory pathway, it is possible that the tumoristatic effect of ATP7A may be the result of reduced cuproenzyme activity. Studies have shown that copper deficiency by either deletion of the CTR1 copper importer, or the use of copper chelators, prevents tumor growth by restricting the MEK1 protein, which has been shown to require copper as a cofactor for ERK phosphorylation [29, 33, 44]. We found no differences in ERK phosphorylation in MEF^{RAS}7A- cells relative to wild type cells (Figure S4), suggesting that the excess

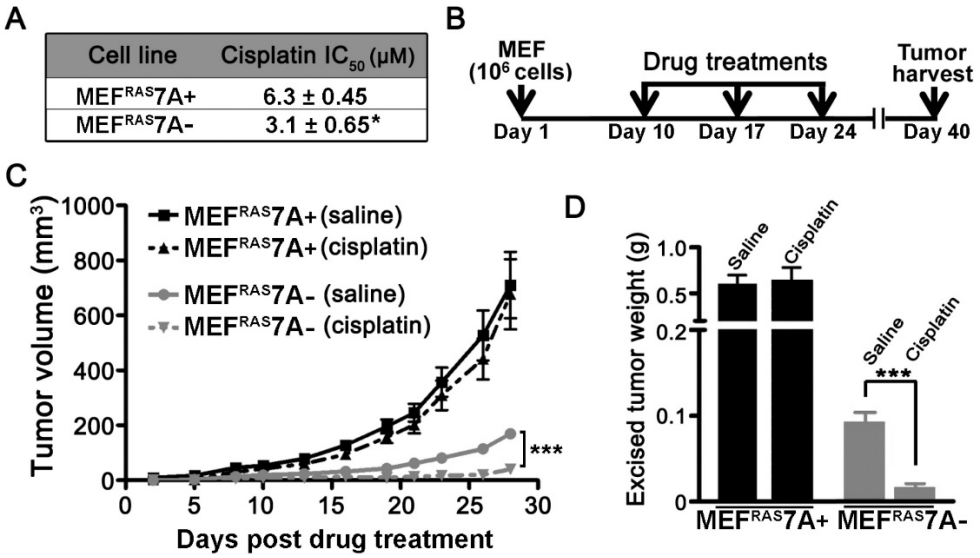

Figure 4. Deletion of *Atp7a* increases cisplatin sensitivity *in vitro* and *in vivo* (A) IC_{50} values for cisplatin were calculated for MEF^{RAS}7A+ and MEF^{RAS}7A- cells exposed to increasing doses of cisplatin for 24 hours. Cell viability was measured using the Prestoblue assay. (B) Schematic illustration of the cisplatin treatment regimen used to assess the role of ATP7A in cisplatin sensitivity in tumor-bearing mice. MEF^{RAS}7A+ and MEF^{RAS}7A- cells were injected subcutaneously into flanks of nude mice. Cisplatin (4mg / kg) or an equal volume of saline control was intraperitoneally injected on days 10, 17 and 24. At day 40, tumors were harvested for tumor volume measurement (C) and weight (D) (n= 10-12 mice per group; mean ± S.E.M., ***P<0.001).

copper accumulation in MEF^{RAS}7A- cells does not increase MEK1 activity. Thus, it would appear from these data that tumor inhibition via perturbation of copper homeostasis may be achieved by restricting copper-dependent pathways for tumor growth or by generating an unfavorable environment for tumor growth by over-accumulating this potentially toxic metal. Previous studies have shown that silencing of ATP7A or ATP7B increases the sensitivity of cultured cells to cisplatin treatment, whereas forced expression of these proteins increases cisplatin resistance [16, 17, 20, 22] [45, 46]. Our results expand on these observations by demonstrating that *ATP7A* deletion can enhance the efficacy of cisplatin treatment *in vivo*. These findings point to the possibility that inhibitors of ATP7A/B might be used in combination with cisplatin to increase efficacy of cisplatin-mediated killing or reverse the resistance to cisplatin that is acquired by increased ATP7A/B expression [16, 21], [47]. In this context it is interesting to note that the proton-pump inhibitor, omeprazole, which was recently shown to block ATP7A-dependent melanogenesis in B16 melanoma cells [48], has been shown to increase the sensitivity of solid tumors to cisplatin chemotherapy in nude mice [49]. While omeprazole is known to affect numerous pathways in cancer cells including extracellular- and lysosomal pH [50], it is tempting to speculate that the potentiation of cisplatin sensitivity by omeprazole may occur via blocking ATP7A activity. The results and strategies described in our study open new avenues of research into the potential use of ATP7A inhibitors in controlling both tumor growth and cisplatin sensitivity.

Supplementary Material

Supplementary figures and methods.
http://www.jcancer.org/v08p1952s1.pdf

Abbreviations

MEF: Mouse embryonic fibroblasts; CTR1: copper transporter 1; CCS: copper chaperone for superoxide dismutase 1; ROS: Reactive oxygen species.

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Ethics approval and consent to participate

Research Involving Animals - All animal husbandry and euthanasia procedures were performed in accordance with, and under the approval of, the Animal Care and Use Committee (ACUC) of the University of Missouri. No human subjects, materials or data have been used in this study.

Availability of supporting data

All data generated or analysed during this study are included in this article and its supplementary information files.

Author contributions

SZ, VS and YW performed experiments. SZ, VS, and MJP wrote the manuscript. JL performed metal measurements and provided technical advice. All authors read and approved the final version of the manuscript.

Competing Interests

The authors declare that they have no competing interests

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