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Title Page

BCRP/ABCG2 Transporter Regulates Accumulation of Cadmium in Kidney Cells: Role of the Q141K Variant in Modulating Nephrotoxicity

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Running Title: BCRP Variant Increases Cadmium Renal Injury

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Abbreviations

BCRP, breast cancer resistance protein; Bcrp KO, Bcrp knockout; Cd, cadmium; CAT, catalase; DMT1, divalent metal transporter 1; EV, empty vector; GSH, glutathione; HEK293, human embryonic kidney 293 cells; HO-1, heme oxygenase; ICP-MS, inductively coupled plasma mass spectrometry; MATE1/2K, multidrug and toxin extrusion protein; MDR1, multidrug resistance protein 1; MT-1A and 2A, metallothionein 1A and 2A; NQO1, NAD(P)H quinone dehydrogenase 1; Nrf2, NF-E2–related factor 2; OCT2, organic cation transporter 2; PI, propidium iodide; ROS, reactive oxygen species; PBS, phosphate-buffered saline; SOD-1, superoxide dismutase 1; TRXR, thioredoxin reductase; WT, wild-type; ZIP8/14, iron and zinc transporter 8/14

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Abstract

Exposure to the environmental pollutant cadmium is ubiquitous as it is present in cigarette smoke and the food supply. Over time, cadmium enters and accumulates in the kidneys where it causes tubular injury. The breast cancer resistance protein (BCRP, *ABCG2*) is an efflux transporter that mediates the urinary secretion of pharmaceuticals and toxins. The *ABCG2* genetic variant Q141K exhibits altered membrane trafficking which results in reduced efflux of BCRP substrates. Here, we sought to 1) evaluate the *in vitro* and *in vivo* ability of BCRP to transport cadmium and protect kidney cells from toxicity, and 2) determine whether this protection is impaired by the Q141K variant. Cadmium concentrations, cellular stress, and toxicity were quantified in HEK293 cells expressing an empty vector (EV), BCRP wild-type (WT), or variant (Q141K) gene. Treatment with $CdCl_2$ resulted in greater accumulation of cadmium and apoptosis in EV cells relative to WT cells. Exposure to $CdCl_2$ induced expression of stress-related genes and proteins including MT-1A/2A, NQO1, and HO-1 to a higher extent in EV cells compared to WT cells. Notably, the Q141K variant protected against $CdCl_2$-induced activation of stress genes and cytotoxicity, but this protection was to a lesser magnitude than observed with WT BCRP. Lastly, concentrations of cadmium in the kidneys of Bcrp KO mice were 40% higher than in WT mice, confirming that cadmium is an *in vivo* substrate of BCRP. In conclusion, BCRP prevents the accumulation of cadmium and protects against toxicity, a response that is impaired by the Q141K variant.

Significance Statement. The BCRP transporter lowers cellular accumulation of the toxic heavy metal cadmium. This protective function is partially attenuated by the Q141K genetic variant in the *ABCG2* gene.

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Introduction

The heavy metal cadmium is a high priority environmental pollutant classified as a chemical of public concern as well as a human carcinogen (World health Organization, 2020). The US ATSDR ranked cadmium seventh on its most recent list of dangerous substances (Agency for Toxic Substances and Disease Registry, 2020, January 17). Cigarette smoking is the most significant source of cadmium exposure, with additional exposure through ingestion of contaminated foods and water (Berglund et al., 1994; Becker et al., 2002; Mannino et al., 2004; reviewed in Satarug and Moore, 2004; reviewed in Mezynska and Brzoska, 2018). As a result, cadmium levels in blood, placenta, and kidneys are consistently higher in smokers than nonsmokers (Becker et al., 2002; Mannino et al., 2004; Kutlu et al., 2006; Ebert-McNeill et al., 2012).

After absorption, the cadmium ion (Cd^{2+}) is rapidly taken up by the liver and kidneys where it forms complexes with the small, cysteine-rich, metal-binding protein metallothionein (MT) or glutathione (GSH) to be released back into the circulation or retained in tissues (Hansen et al., 2006; Leverrier et al., 2007; Klaassen et al., 2009). Once in the circulation, Cd^{2+}-MT can be readily filtered by the glomerulus and reabsorbed by proximal tubules using endocytosis (Zalups, 2000; Zalups and Barfuss, 2002). Cadmium exposure induces MT production by the liver and kidneys, which is considered a protective response to prevent the toxicity from free Cd^{2+} (Liu et al., 1995; Park et al., 2001). With chronic exposure, most Cd^{2+} accumulates in the kidneys due to filtration and uptake mediated by transporters (Park et al., 2002; Satarug et al., 2002; Uetani et al., 2006; Soodvilai et al., 2011; Fujishiro et al., 2012). Uptake transporters include the organic cation transporter 2 (OCT2), divalent metal transporter 1 (DMT1) and zinc/iron protein 8/14

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(ZIP8/14). Therefore, as a result of active uptake, the kidneys are considered to be a target organ by chronic Cd^{2+} toxicity.

Cd^{2+}-mediated nephrotoxicity is multifactorial and involves direct DNA damage, oxidative stress and lipid peroxidation, and depletion of intracellular GSH levels (Liu and Jan, 2000; Ikediobi et al., 2004; Lopez et al., 2006). Moreover, Cd^{2+} inhibits the mitochondrial electron transport chain, resulting in generation of reactive oxygen species (ROS), mitochondrial dysfunction, and activation of protein kinases that trigger cellular stress or death (Thevenod and Friedmann, 1999; Ikediobi et al., 2004; Shih et al., 2004). Cd^{2+} also has high affinity for sulfhydryl groups of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glucose-6-phosphate dehydrogenase (G6PD), leading to inhibition of their antioxidant activity (Geret et al., 2002; Kasperczyk et al., 2012). To combat these responses, cells activate transcription factors sensitive to redox signaling including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1) and NF-E2–related factor 2 (Nrf2) to limit the extent of Cd^{2+}-induced ROS and kidney injury (Casalino et al., 2007; Thijssen et al., 2007; He et al., 2008). Therefore, tissue tolerance mechanisms enable adaptation to Cd^{2+} toxicities (Thijssen et al., 2007; reviewed in Nemmiche, 2017).

Several transporters including the multidrug resistance protein 1 (MDR1) and multidrug and toxin extrusion protein (MATE1/2K) localized in the luminal membrane of kidney proximal tubule cells have been reported to efflux Cd^{2+} and/or thiol-conjugated complexes *in vitro* (Kimura et al., 2005; Yang et al., 2017). In addition to these transporters, BCRP (breast cancer resistance protein, *ABCG2*) is also expressed in the kidneys where it facilitates the urinary secretion of drugs and toxins (reviewed in Klaassen and Aleksunes, 2010; reviewed in George et al., 2017). Prior studies have suggested that BCRP may efflux Cd^{2+} and protect cells from

subsequent injury. Following exposure of pregnant rats to Cd^{2+}, BCRP protein is down-regulated in the placenta (Liu et al., 2016). Likewise, in human placental BeWo cells and MCF-7 cells that overexpress BCRP, Cd^{2+} inhibits the efflux of BCRP substrates (Kummu et al., 2012). It is important to note that BCRP exhibits interindividual variation due to the nonsynonymous polymorphism C421A (Q141K) in the *ABCG2* gene. The Q141K variant has been associated with altered pharmacokinetics of the chemotherapeutic drug gefitinib and hypolipidemic drug rosuvastatin (Cusatis et al., 2006; Keskitalo et al., 2009b). Cells expressing the Q141K variant have reduced *in vitro* efflux of substrates compared to cells expressing wild-type BCRP (Xiao et al., 2015; Bircsak et al., 2016). In the present study, we sought to characterize the ability of BCRP to transport Cd^{2+} from kidney cells and prevent subsequent toxicity. In addition, the effect of the genetic variant Q141K on Cd^{2+} transport mediated by BCRP was also evaluated *in vitro*.

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Materials and Methods

Reagents and Chemicals. Unless specified, chemicals and reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Human embryonic kidney 293 (HEK293) cells stably expressing an empty vector (EV, pcDNA 3.1), the wild-type (WT) human BCRP/*ABCG2* gene, or the C421A BCRP/*ABCG2* variant were generously provided by Dr. Robert Robey from the National Cancer Institute at the National Institutes of Health in Bethesda, MD (Morisaki et al., 2005; Bircsak et al., 2016). Cells were cultured in Dulbecco's modified Eagle medium (ThermoFisher Scientific, Carlsbad, CA) supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 0.2 mg/ml geneticin (ThermoFisher Scientific).

Cadmium Accumulation. HEK293 EV, BCRP WT, and BCRP Q141K cells ($1x10^6$/well) were seeded on poly-D-lysine coated 6-well plates (Fisher Scientific, Pittsburgh, PA) overnight and treated with cadmium chloride ($CdCl_2$, 0.1-1 µM) for 30 min (*uptake period*). In humans exposed to cadmium through the environment, the mean circulating concentration of cadmium was ~20 nM (Staessen et al., 1994; Olsson et al., 2002; Chen et al., 2006). However, concentrations as high as 0.12-0.5 µM have been observed in smokers and individuals exposed occupationally (Hassler et al., 1983; Nishijo et al., 2004). Therefore, we selected *in vitro* concentrations within the low micromolar range (0.5 and 1 µM) for the present study. Following washing cells with phosphate-buffered saline (PBS), untreated culture media was added for 60 min (*efflux period*). Following washing and centrifugation, cell pellets were incubated with lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, and 1% Triton-100) on ice for 10 min. Following lysis of cells, supernatants were obtained by centrifugation (9000g x 2 min) and used for quantification of cadmium accumulation (inductively coupled plasma-mass spectrometry,

ICP-MS analysis) and normalized to concentration of protein (bicinchoninic acid, BCA, protein assay).

Animal Treatment. Wild-type (WT) C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Bcrp knockout (KO) mice were obtained from Taconic Biosciences (Taconic, NY) and backcrossed to the 027 C57BL/6 strain (Charles River Laboratories, Wilmington, MA) until over 99% congenic (Rutgers RUCDR Infinite Biologics, Piscataway, NJ). Groups of 13- to 20-week-old adult WT (n= 7-10) and Bcrp KO (n= 9-12) male and female mice were administered (i.p.) a single dose of either 5 ml/kg of saline vehicle (0.9% NaCl) or 5.5 mg/kg of $CdCl_2$. The *in vivo* dose (5.5 mg/kg $CdCl_2$) was chosen based off previous literature (Li et al., 2011; Micali et al., 2018; Chen et al., 2021) with a goal of sufficient detection using ICP/MS. Kidneys and plasma were collected at 24 h after $CdCl_2$ administration. Tissues were snap frozen and stored at −80ºC. Renal and plasma cadmium concentrations in WT and Bcrp KO mice were quantified by ICP-MS analysis. The Institutional Animal Care and Use Committee approved these experiments under protocol #09-037.

ICP-MS analysis. Intracellular cadmium concentrations were quantified using ICP-MS analysis. In brief, samples (50 µl) were mixed with concentrated ultra high-purity nitric acid (100 µl, VWR, Radnor, PA) and sonicated for 60 min, followed by digestion using a CEM microwave system (CEM, Matthews, NC) for 30 min at 300 W (50-75% power). Samples were diluted with MilliQ water to produce an acid concentration of 5% HNO_3 for analysis via ICP-MS (approx. dilution factor = 40x). Cadmium concentrations in digested samples were quantified using a Nu AttoM high resolution ICP-MS, at low resolution (300). Conditions were operated at RF power of 1550 W, carrier gas flow of 1 L/min Ar, and nebulizer gas flow of ~36 psi Ar. Three replicates of masses ^{110}Cd, ^{111}Cd, and ^{112}Cd were quantified in deflector jump mode with 5 ms peak dwell

time, 250 sweeps, and 2 cycles, and averaged (RSD <5%). Calibration standards were prepared each day with cadmium concentrations ranging between 0.001-10 ppb, in 5% HNO_3, with an instrument detection limit <0.005 ppb. Sample concentrations were extrapolated using a linear regression through at least 5 standards, with a correlation coefficient > 0.999 for all runs. Quality control standard (NIST A) were quantified after every sixth sample to account for instrument drift and monitor reproducibility, and reproduced with RSD <5% (n=4-15 dependent on batch size). Matrix matched standards (cadmium-spiked control) were measured to account for matrix effects (91-103% yield).

Cell Viability and Cytotoxicity Assays. Cells ($5x10^3$/well) were seeded in poly-D-lysine coated 96-well plates overnight and exposed to increasing concentrations of $CdCl_2$ (0-10 μM) for 48 h. $CdCl_2$ is readily soluble in cell culture media. Cell viability and cytotoxicity were determined using the alamarBlue and propidium iodide (PI) staining assays, respectively. AlamarBlue reagent is a redox indicator dye that evaluates the metabolic activity of cells. After treatment of cells with vehicle or $CdCl_2$, alamarBlue reagent (resazurin, dissolved in PBS) was added and incubated with culture media for 3 h. Following incubation, fluorescence was measured at 570/585 nm (excitation/emission) using a Spectramax M3 microplate reader (Molecular Devices, San Jose, CA). The percentage of viable cells was calculated by comparing fluorescence reading for each treatment relative to control cell (no $CdCl_2$ treatment). PI is a fluorescent dye that is not permeable across the plasma membrane of viable cells, and in turn, is routinely used to detect and quantify dead or dying cells. Following treatment with $CdCl_2$ (0-10 μM), cells were stained with PI (1 μg/ml, Sigma) and Hoechst 33342 (3 μM, a fluorescent stain for labeling DNA, Nexcelom Bioscience, Lawrence, MA). The percentage of dead cells (PI-stained) was calculated

using a Celigo Imaging Cytometer (Nexcelom Bioscience) and normalized to the total number of cells stained with Hoechst 33342.

RNA Isolation and mRNA Quantification. $CdCl_2$-treated cells (0-1 µM, 24 h) or the kidneys of WT and Bcrp KO mice were homogenized using a TissueLyser LT (Qiagen, Valencia, CA). Total RNA was isolated using RNAzol RT (Sigma) according to the manufacturer's protocol. The concentration of total RNA was measured by UV spectrophotometry (260/280 nm) using a Nanodrop spectrophotometer 2000 (Thermo Fisher Scientific). Messenger RNA expression of human HO-1 (heme oxygenase-1), NQO1 (NAD(P)H quinone oxidoreductase 1), MT-1A/2A (metallothionein 1A/2A), SOD-1, TRXR (thioredoxin reductase), DMT1, ZIP8/14 or mouse Bcrp, Mate1, Mdr1a/1b (multidrug resistance protein 1a/1b), Oct2, Dmt1, and Zip8/14 was quantified by qPCR assay using Sybr Green to detect amplified products in an ABI ViiA 7 system (Applied Biosystems, Carlesbad, CA). Ct values were converted to ΔΔCt by normalizing to reference genes, human 18S or mouse ribosomal protein L13a (Rpl13a). Primers are listed in Supplementary Tables 1 and 2.

Protein Isolation and Western Blot Analysis. Cells were treated with $CdCl_2$ (0-1 µM) for 24 h and lysed in buffer containing 20 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton 100 and 1% protease inhibitor cocktail. Protein concentrations of cell lysates were quantified using the BCA protein assay kit (ThermoFisher Scientific). For Western blotting, cell lysates (15-25 µg of protein/sample) were separated by SDS-PAGE and transferred overnight (4°C) onto a nitrocellulose membrane. After blocking with 5% nonfat dry milk in PBS with 0.5% Tween 20 (PBS/T), membranes were incubated with primary antibodies against BCRP (ALX-801-036-C100), HO-1 (ADI-SPA-895-F) (Enzo Life Sciences, Farmingdale, NY), NQO1 (ab80588), MT1/2 (ab12228), SOD-1 (ab13498), or TRXR (ab18840) (Abcam, Cambridge, MA) followed

by incubation with species-appropriate secondary antibodies for 1 to 2 h. SuperSignal West Dura chemiluminescent substrate (ThermoFisher Scientific) was added to membranes in order to detect luminescence on a FluorChem E system (Protein Simple, San Jose, CA). Intensity of target protein bands was semi-quantified and normalized to Na^+/K^+ ATPase (ab76020, Abcam, Cambridge, MA) levels.

Data Analysis. The concentration of $CdCl_2$ at half-maximal inhibition for cell viability (LC_{50}) was calculated using nonlinear regression best curve fitting analysis (GraphPad Prism 6.0 Software, San Diego, CA). Identification of statistical outliers within treatment groups was performed using GraphPad QuickCalcs. Individual data were expressed as mean ± SD and analyzed by t-test or two-way ANOVA with Newman-Keuls multiple comparison test using GraphPad Prism software (version 6; GraphPad Software Inc). Significance was set at $p < 0.05$ for all analyses.

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Results

Concentration-Dependent Accumulation and Toxicity of $CdCl_2$ in HEK293 Empty Vector (EV) and BCRP Wild-type (BCRP WT) Cells. We have previously characterized the expression and function of BCRP in HEK293 cells transfected with EV and human BCRP/*ABCG2* WT gene (Xiao et al., 2015; Bircsak et al., 2016; Szilagyi et al., 2019). To test whether $CdCl_2$ is a novel substrate of BCRP, the intracellular concentration of cadmium was quantified in EV and BCRP WT cells following $CdCl_2$ treatment (Fig 1A). Compared to EV cells, BCRP WT cells had 30-50% lower intracellular cadmium accumulation at each concentration of $CdCl_2$ tested. Consistent with this finding, the lower cadmium accumulation in BCRP WT cells resulted in higher cell viability (Fig 1B) and less extensive cell death (Fig 1C). Using the alamarBlue assay, the LC_{50} for $CdCl_2$-induced cytotoxicity at 48 h in BCRP WT cells was 24 μM, which was 6-fold higher than that calculated for EV cells (3.6 μM). Overexpression of BCRP decreased apoptosis in BCRP WT cells with a 50-80% reduction in PI staining compared to EV cells.

The transporters DMT1 and ZIP8/14 mediate uptake of Cd^{2+} across the apical side of proximal tubule cells (Park et al., 2002; Fujishiro et al., 2012). We quantified mRNA levels of both transporters in EV and BCRP WT cells and observed no differences in expression (Supplementary Fig 1). Expression of MDR1 protein, a previously identified Cd^{2+} efflux transporter (Kimura et al., 2005), was undetectable by Western blot analysis in both cell lines as well (data not shown).

Concentration-Dependent Up-Regulation of Oxidative and Cellular Stress Pathways in EV and BCRP WT Cells Treated with $CdCl_2$. Oxidative stress is one mechanism implicated in

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the pathogenesis of Cd^{2+}-induced hepatic and renal toxicity (reviewed in Shaikh et al., 1999; Nemmiche, 2017). For these experiments, EV and BCRP WT cells were treated with two different concentrations of $CdCl_2$ (0.5 and 1 μM) for 24 h and expression of genes involved in cellular defense and detoxification against oxidative (HO-1, NQO1, SOD-1, TRXR) and metal stress (MT-1A, MT-2A) were quantified using qPCR (Fig 2A). In EV cells, expression of cytoprotective genes HO-1 and NQO1 showed a concentration-dependent increase, whereas no change was observed in BCRP WT cells. In response to heavy metal exposure, cells typically up-regulate the expression of metal sequestration genes MT-1A/2A. As expected, $CdCl_2$ exposure led to varying increase in MT-1A and 2A mRNAs in both EV and BCRP WT cells. In response to 1 μM $CdCl_2$ treatment, the mRNA expression of MT-1A and 2A was up-regulated over 20-fold and 100-fold, respectively, in EV cells. However, in BCRP WT cells, induction of MT-1A (10-fold) and MT-2A (25-fold) was lower. The mRNA expression of other antioxidant enzymes, SOD-1 and TRXR, was not remarkably modulated by $CdCl_2$ treatment in either cell line.

Regulation of oxidative and cellular stress-related proteins in response to $CdCl_2$ for 24 h was also assessed (Fig 2B). As expected, BCRP protein was only expressed in BCRP WT cells, and not in EV cells. Consistent with data shown in Fig 2A, $CdCl_2$ treatment increased HO-1, NQO1, MT-1/2 and SOD-1 protein expression in a concentration-dependent manner in EV cells, but not in BCRP WT cells. Interestingly, lower basal levels of HO-1 protein were observed in BCRP WT cells. Despite no changes in mRNA levels at 24 h (Fig 2A), the protein expression of TRXR was similarly up-regulated by $CdCl_2$ (1 μM) in both EV and BCRP WT cells.

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$CdCl_2$ Accumulation and Toxicity in BCRP Q141K Cells. The Q141K polymorphism in the *ABCG2/*BCRP gene reduces efflux of some BCRP substrates (Xiao et al., 2015) and has been associated with altered pharmacokinetics in patients (Cusatis et al., 2006; Keskitalo et al., 2009b; Bircsak et al., 2018). We and others have previously shown that overexpression of the BCRP Q141K gene decreases the extent of *in vitro* efflux for two BCRP substrates, the mycoestrogen zearalenone and the dye Hoechst 33342 (Xiao et al., 2015; Bircsak et al., 2016) (Supplementary Fig 2). Thus, through further experiments we aimed to determine whether the Q141K variant alters efflux of Cd^{2+} and in turn, its toxicity compared to WT BCRP. Similar to data shown in Fig 1A, overexpression of WT BCRP in HEK293 cells reduced cadmium accumulation by 40% (Fig 3A). By comparison, cells expressing the Q141K variant, exhibited only a 20% reduction of cadmium accumulation compared to HEK EV cells. Consequently, the LC_{50} for $CdCl_2$ treatment in BCRP Q141K cells was 10.9 µM at 48 h, which was 2-fold lower than that observed in BCRP WT cells (21.8 µM), and 1.5-fold higher than EV cells (Fig 3B). In addition, the extent of $CdCl_2$-induced apoptosis was greatest in EV cells followed by BCRP Q141K cells with BCRP WT cells exhibiting the lowest sensitivity (Fig 3C).

Oxidative and Cellular Stress Gene and Protein Expression in BCRP Q141K Cells Treated with $CdCl_2$. Subsequent studies assessed the ability of cells expressing the BCRP Q141K variant to up-regulate cellular stress genes and proteins in response to $CdCl_2$ treatment (Fig 4). Consistent with data shown in Fig 2, $CdCl_2$ (1 µM) increased HO-1, NQO1, MT-1A/2A mRNA levels in EV cells, with little to no change in expression observed in BCRP WT cells. Notably, BCRP Q141K cells treated with $CdCl_2$ did exhibit up-regulation of NQO1, MT-1A, and MT-2A mRNAs.

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Total expression of BCRP protein in cell lysates was similar in BCRP Q141K and WT cells as we have previously reported (Xiao et al., 2015; Bircsak et al., 2016) (Fig 4B). As expected, $CdCl_2$ increased the protein expression of HO-1, NQO1, and MT-1/2 (5-fold, 2-fold, and 20-fold, respectively) in EV cells, but in BCRP WT cells, $CdCl_2$ only modestly induced MT protein expression by 1.5-fold with no change in HO-1 or NQO1 levels (Fig 4B). Following $CdCl_2$ treatment, the protein expression of HO-1, NQO1, and MT-1/2 was also up-regulated in BCRP Q141K cells (2-fold, 0.5-fold and 6-fold, respectively). Importantly, the protein expression of HO-1, NQO1, and MT-1/2 in BCRP Q141K cells was increased to a higher magnitude than that observed in BCRP WT cells (Fig 4B).

Renal and Plasma Cd^{2+} Concentrations in WT and Bcrp KO Mice. Bcrp is highly expressed in both male and female rodent kidneys (Tanaka et al., 2005). To determine whether Bcrp can transport Cd^{2+} *in vivo*, concentrations of cadmium were quantified in kidneys and plasma of WT and Bcrp KO mice at 24 h after a single administration (ip, 5.5 mg/kg) (Fig 5). There was no difference in cadmium concentrations observed between sexes within each genotype and are shown together. No notable difference in cadmium plasma concentrations was observed in WT and Bcrp KO mice, suggesting similar distribution between the two genotypes. By comparison, concentrations of cadmium in the kidneys of Bcrp KO mice were 40% higher than in WT mice consistent with the *in vitro* data. Notable, the mRNA expression of other cadmium uptake (Oct2, Dmt1, and Zip8/14) and efflux transporters (Mate1 and Mdr1a/1b) was similar between genotypes (Supplementary Fig 3).

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Discussion

In the present study, we demonstrated *in vitro* that cells expressing BCRP had lower intracellular cadmium accumulation, resistance to $CdCl_2$-induced cytotoxicity, and lesser induction of stress-related gene expression compared to control cells expressing EV. Consistently, concentrations of cadmium in the kidneys of Bcrp KO mice were higher than in WT mice, indicating that BCRP participates in the efflux of Cd^{2+} in kidneys, and can potentially protect against Cd^{2+} toxicity. In addition, the BCRP gene variant Q141K suppressed $CdCl_2$-induced activation of stress genes and cytotoxicity compared to the EV cells, but the extent of decrease was less than that observed with the WT BCRP protein. Therefore, the reduced function variant Q141K in BCRP/*ABCG2* results in greater Cd^{2+} accumulation and heightened susceptibility to renal cell injury.

BCRP has multiple drug binding sites, and transports a wide range of structurally diverse substrates including chemotherapeutic agents, organic anion conjugates and chemical toxicants. In kidney, Cd^{2+} and its complexes (Cd^{2+}-MT, Cd^{2+}-GSH or cysteine) are filtered, and reabsorbed into the proximal tubular cells via endocytosis or transport (Dudley et al., 1985; Soodvilai et al., 2011). However, the current ICP/MS method is unable to resolve whether Cd is conjugated to other molecules. Future studies are needed to identify the exact Cd species or conjugates transported by BCRP.

After chronic exposure to low concentrations of environmental or dietary cadmium, Cd^{2+} is absorbed from the gastrointestinal tract, and largely binds to albumin and other thiol-containing proteins such as GSH, cysteine, or homocysteine in the circulation, and is transported to the liver. MTs are small cysteine-rich proteins that contribute to metal homeostasis and protect

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against the toxicity of heavy metals including DNA damage and oxidative stress. In humans, MTs have four main isoforms: MT-1, MT-2, MT-3, and MT-4. The major isoforms, MT-1 and MT-2, are present in most tissues (reviewed in Takahashi, 2012; reviewed in Ruttkay-Nedecky et al., 2013). In the liver, Cd^{2+} rapidly induces the synthesis of endogenous MTs, which in turn bind to and sequester Cd^{2+}, thereby limiting its toxicity (Liu et al., 1995; Park et al., 2001; Klaassen et al., 2009). The conjugated and bound forms of cadmium are not toxic by themselves, but the free divalent form of Cd (Cd^{2+}) liberated from the complexes imparts cellular toxicity (Barbier et al., 2005). In the current study, MT-1/2 protein was undetectable in unstimulated control cells. After cells were treated with $CdCl_2$ (1 μM) for 24 h, MT-1A/2A mRNA and protein expression was dramatically induced, with up-regulation of gene expression over 20-fold and 100-fold for MT-1A and 2A, respectively, in EV cells. However, in the cells expressing WT BCRP protein, induction of MT-1A (10-fold) and MT-2A (25-fold) gene expression was attenuated. Due to the ability of BCRP WT cells to efficiently lower cadmium intracellular accumulation, the need to activate cellular protective mechanisms such as MTs synthesis was reduced.

Cd^{2+} is a toxic metal ion that can compete with essential metal ions such as Fe^{2+}, Zn^{2+}, Cu^{2+} or Ca^{2+} and once inside cells, initiate free radical chain reactions by blocking mitochondrial electron transport, deplete endogenous redox scavengers, and inhibit antioxidant enzymes culminating in cell death (reviewed in Patra et al., 2011; reviewed in Thevenod and Wolff, 2016). Over the past two decades, Nrf2 has become recognized as a key cellular defense system that counteracts oxidative stress, apoptosis, and inflammation. Cadmium activates Keap1/Nrf2 signaling and up-regulates downstream heat shock and antioxidant enzymes HO-1 and Glutamate-Cysteine Ligase Catalytic Subunit (GCLC), leading to protection against Cd^{2+}-induced apoptosis in rat kidney cells and mouse hepatoma cells (He et al., 2008; Chen and

Shaikh, 2009). Following exposure of male chickens to Cd^{2+}, a coordinated activation of Nrf2 target genes (NQO1, HO-1, SOD, CAT and GCLC) was evident within the kidneys (Ge et al., 2019). In the present study, in response to low concentrations of $CdCl_2$ (0.5 and 1 μM), the mRNA and protein expression levels of HO-1 and NQO1 were remarkably increased in EV cells, whereas no change was observed in BCRP WT cells. These findings are consistent with the lower intracellular accumulation of cadmium owing to the ability of BCRP to efflux Cd^{2+}. This in turn resulted in less activation of Nrf2 signaling in kidney cells expressing BCRP. These data point to cellular efflux by BCRP as a key initial defense mechanism that can reduce the need to activate intracellular defenses such as Nrf2.

One of the earliest insights into BCRP function was its ability to interact with porphyrins such as heme. HO-1 is a heat shock protein inducible by Nrf2 as well as the rate-limiting enzyme involved in heme degradation. Expression of HO-1 is also inducible in the presence of heme (Turner et al., 1998; Immenschuh and Ramadori, 2000). In the current study, basal mRNA and protein expression of HO-1 was notably lower in cells expressing BCRP protein. This is presumably a result from BCRP efflux of heme leading to reduced intracellular levels (Jonker et al., 2002; Krishnamurthy et al., 2004).

BCRP localizes to the apical membrane of renal proximal tubules where it participates in the renal excretion of xenobiotics, dietary mutagens, and carcinogens (reviewed in Nakanishi and Ross, 2012; reviewed in Jani et al., 2014). BCRP is markedly expressed in mouse kidneys (Tanaka et al., 2005; Huls et al., 2008) and immunostaining demonstrates its enrichment in the brush-border membrane of cortical proximal tubules (Huls et al., 2008). However, the relative

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abundance of BCRP expression in human kidneys is less clear as contradictory reports have been published by different laboratories. In healthy human kidneys, low mRNA levels of BCRP were found, and the protein expression was almost undetectable by western blot with the monoclonal BCRP antibody BXP-21 (Doyle and Ross, 2003). Consistently, by using quantitative targeted proteomics, BCRP protein was detected in human kidney cortex, but at levels below the lower limit of quantification (Prasad et al., 2016; Oswald et al., 2019). However, in another study, Huls et al., reported detectable BCRP expression in human kidney membranes and in the isolated human proximal tubular cells using western blot analysis with the BXP-9 antibody. Likewise, immunofluorescent staining with the BXP-9 antibody revealed BCRP in the brush border membranes of human proximal tubules (Huls et al., 2008). Furthermore, efflux of the Hoechst 33342 dye from primary human proximal tubule cells was impaired by BCRP inhibitors, fumitremorgin C and nelfinavir (Huls et al., 2008). In addition to this study, quantifiable BCRP protein expression, albeit low, has been demonstrated using nanoLC-MS/MS (Fallon et al., 2016). Recognizing these conflicting results, it would be prudent to determine whether BCRP can efflux cadmium from primary human proximal tubule epithelial cells similar to what was observed in overexpressing cells as well as mouse kidneys.

Several *ABCG2* polymorphisms have been identified and associated with alterations in BCRP expression/function both *in vitro* and *in vivo* (Kobayashi et al., 2005; Furukawa et al., 2009; Woodward et al., 2013). Among these genetic variants, the Q141K (C421A) is the most well-studied and characterized, and found at allele frequencies around 30% in Asians and 10% in Caucasians (Zamber et al., 2003; Kobayashi et al., 2005; Bircsak et al., 2018). In HEK293 cells expressing Q141K, the efflux of the BCRP substrate glyburide was reduced compared to cells

expressing WT BCRP (Pollex et al., 2010). Murine fibroblast PA317 cells transfected with the BCRP Q141K variant showed remarkably decreased protein expression and greater topotecan accumulation compared with WT cells (Imai et al., 2002). Several clinical studies have also revealed that individuals expressing the Q141K variant experience altered pharmacokinetics and increased risk of drug-related adverse effects (reviewed in Giacomini et al., 2013; reviewed in Hira and Terada, 2018). For example, individuals heterozygous for Q141K exhibit a 22% reduction in the apparent clearance of the BCRP substrate and anticancer drug imatinib compared with patients homozygous for the reference allele (Petain et al., 2008). Likewise, the estimated area under the plasma concentration-time curve from 0 h to infinity (AUC0–∞) of the BCRP substrate and hypolipidemic drug fluvastatin was larger in participants with the A/A variant genotype than in those with the C/A or C/C genotype (Keskitalo et al., 2009a). Our previous studies have also demonstrated that BCRP protein expression levels in the cell surface of HEK293 cells transfected with Q141K decreased by 50% compared to the WT cells (Bircsak et al., 2016). The variant Q141K corresponded with 40%–50% lower BCRP protein in the 421C/A and 421A/A human term placentas compared with WT (421C/C) (Bircsak et al., 2018) and reduced *in vitro* efflux of zearalenone (Xiao et al., 2015). Consistent with these previous studies, the current study revealed higher cadmium accumulation and greater cytotoxicity in HEK293 transfected with the BCRP Q141K variant compared to WT BCRP. In turn, cells expressing BCRP Q141K up-regulated alternate cytoprotective pathways (notably, NQO1, MT-1A, and MT-2A) compared to cells capable of cadmium efflux by WT BCRP. Therefore, the reduced function Q141K variant in the BCRP/*ABCG2* transporter may not confer sufficient protection against Cd^{2+}-induced injury *in vivo*. While this study focused largely on Cd^{2+} efflux by

kidney cells, the findings likely have broader impact as BCRP is expressed in the intestinal tract, brain, and placenta.

In summary, the lower cadmium accumulation and resistance of BCRP WT cells to $CdCl_2$ cytotoxicity indicate that BCRP participates in the cellular efflux of Cd^{2+} and protects against injury. However, this protective response is limited by the reduced function Q141K variant.

Authorship Contributions

Participated in research design: Wen, Aleksunes

Conducted experiments: Wen, Kozlosky, Zhang, Doherty

Contributed new reagents or analytic tools: Buckley, Aleksunes

Performed data analysis: Wen, Kozlosky, Doherty, Buckley

Wrote or contributed to the writing of the manuscript: Wen, Barrett, Aleksunes

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Footnotes

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Figure Legends

***Figure 1. Concentration-Dependent Accumulation and Toxicity of $CdCl_2$ in HEK293 Empty Vector (EV) and BCRP Wild-type (BCRP WT) Cells*.** **(A)** EV and BCRP WT cells were treated with $CdCl_2$ (0-1 μM) for 30 min (*uptake period*), washed, and then incubated in fresh culture media for 60 min (*efflux period)*. Intracellular cadmium concentrations were quantified using ICP-MS analysis and normalized to protein concentration. **(B)** EV and BCRP WT cells were treated with $CdCl_2$ (0-25 μM) for 48 h. Cell viability was determined using the alamarBlue assay. **(C)** EV and BCRP WT cells were treated with $CdCl_2$ (0-10 μM) for 48 h. Cells were stained with propidium iodide (PI) and Hoechst 33342. The number of apoptotic cells (PI-stained) was counted using a Celigo Imaging Cytometer and expressed as a percentage relative to the total number of cells with nuclei stained by Hoechst 33342. Individual data are presented as mean ± SD (n= 3-5). * $p < 0.05$, compared to EV or BCRP WT control (0 or 0.1 μM $CdCl_2$). † $p < 0.05$, compared to EV cells treated with the same concentration of $CdCl_2$.

Figure 2. Concentration-Dependent Up-Regulation of Oxidative and Cellular Stress Gene and Protein Expression in EV and BCRP WT Cells Treated with $CdCl_2$. EV and BCRP WT cells were treated with $CdCl_2$ (0, 0.5, and 1 μM) for 24 h. **(A)** Total RNA was isolated using RNAzol RT. The mRNA expression of HO-1, NQO1, MT-1A, MT-2A, SOD-1 and TRXR was quantified by qPCR assay using Sybr Green to detect amplified products. Ct values were converted to ΔΔCt by comparing to the reference gene 18S. Individual data are presented as mean ± SD (n=6-9). **(B)** Protein expression of BCRP, HO-1, NQO1, MT-1/2, SOD-1 and TRXR in cell lysates was detected and semi-quantified using Western blot analysis. Na^+/K^+ ATPase was used as a loading control. Individual data are presented as mean ± SD (n= 3). * $p < 0.05$, compared to 0 μM $CdCl_2$. † $p < 0.05$, compared to EV cells treated with the same concentration of $CdCl_2$.

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Figure 3. $CdCl_2$ Accumulation and Toxicity in BCRP Q141K Cells. **(A)** EV, BCRP WT, and BCRP Q141K cells were treated with $CdCl_2$ (0.5 µM) for 30 min (*uptake period*), washed, and then incubated in fresh culture media for 60 min (*efflux period*). Intracellular cadmium concentrations were determined using ICP-MS analysis and normalized to protein concentration. **(B)** EV, BCRP WT, and BCRP Q141K cells were treated with $CdCl_2$ (0-25 µM) for 48 h. Cell viability was determined using the alamarBlue assay. **(C)** EV, BCRP WT, and BCRP Q141K cells were treated with $CdCl_2$ (0-10 µM) for 48 h. Cells were then stained with PI and Hoechst 33342. The number of apoptotic cells (PI-stained) were counted using a Celigo Imaging Cytometer and expressed as a percentage relative to the total number of cells with nuclei stained by Hoechst 33342. Individual data are presented as mean ± SD (n= 4-6). * $p < 0.05$, compared to EV, BCRP WT, or BCRP Q141K control (0 µM or 0.1 µM of $CdCl_2$). † $p < 0.05$, compared to EV cells treated with the same concentration of $CdCl_2$. & $p < 0.05$ compared to BCRP WT cells treated with the same concentration of $CdCl_2$.

Figure 4. Oxidative and Cellular Stress Gene and Protein Expression in BCRP Q141K Cells Treated with $CdCl_2$. EV, BCRP WT, and BCRP Q141K cells were treated with $CdCl_2$ (0 and 1 µM) for 24 h. **(A)** Total RNA was isolated using RNAzol RT. The mRNA expression of HO-1, NQO1, MT-1A, MT-2A, SOD-1 and TRXR was quantified by qPCR assay using Sybr Green to detect amplified products. Ct values were converted to ΔΔCt by comparing to the reference gene 18S. Individual data are presented as mean ± SD (n=6-9). **(B)** Protein expression of BCRP, HO-1, NQO1, MT-1/2, SOD-1 and TRXR in cell lysates was detected and semi-quantified using Western blot analysis. Na^+/K^+ ATPase was used as a loading control. Individual data are

presented as mean ± SD (n= 3). * $p < 0.05$, compared with 0 μM $CdCl_2$. † $p < 0.05$, compared with the corresponding EV cells with 1 μM $CdCl_2$ treatment. & $p < 0.05$ compared to BCRP WT cells treated with 1 μM $CdCl_2$.

Figure 5. Renal and plasma Cd^{2+} concentrations in WT and Bcrp KO mice. WT and Bcrp KO mice were treated with $CdCl_2$ (5.5 mg/kg, ip). Kidney and plasma were collected at 24 h and cadmium concentration was quantified using ICP-MS analysis. No differences in cadmium concentrations were observed between sexes within each genotype and are shown together. Data from each individual animal are presented along with mean ± SD (n= 7-12). * $p < 0.05$, compared to WT mice.

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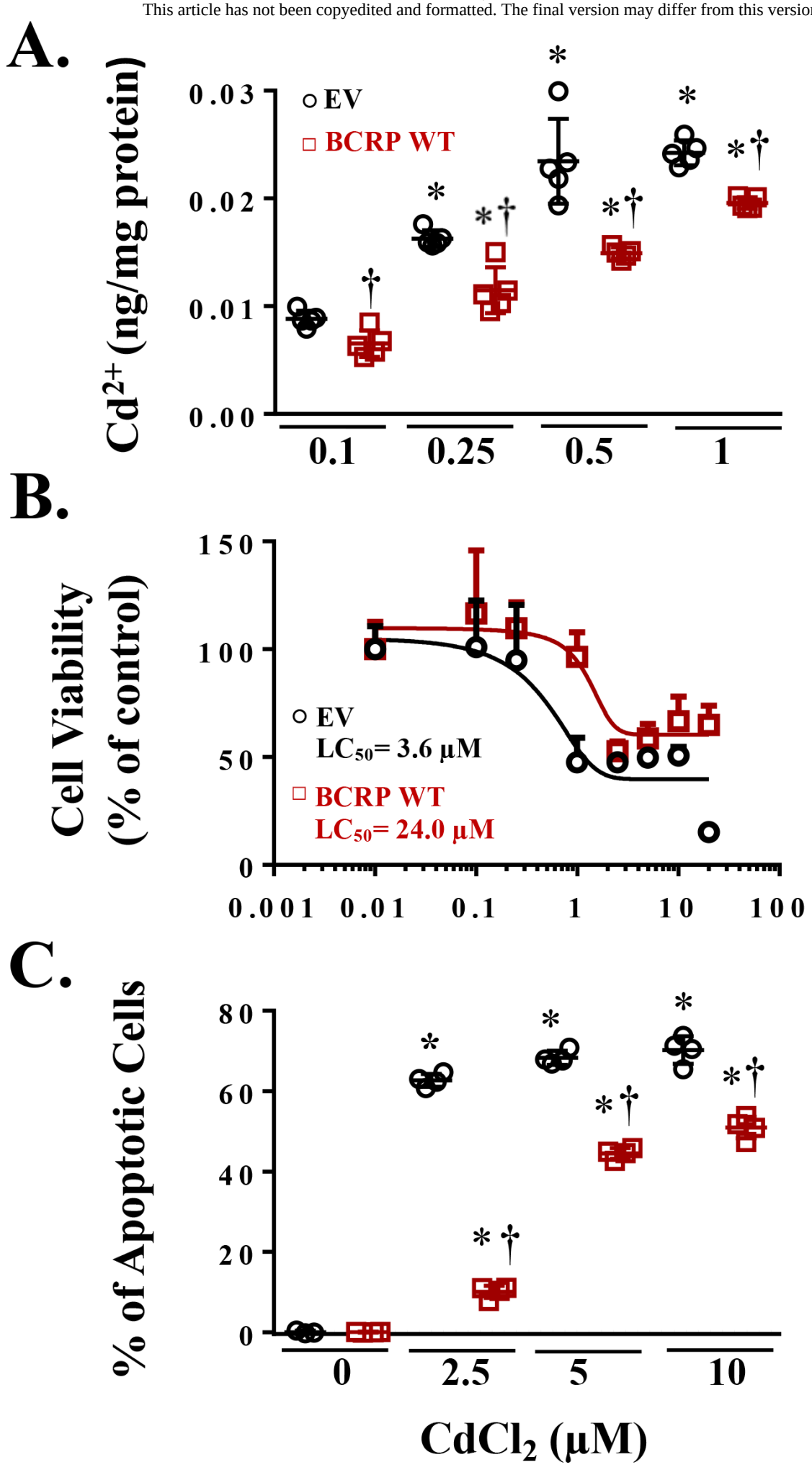

Fig 1

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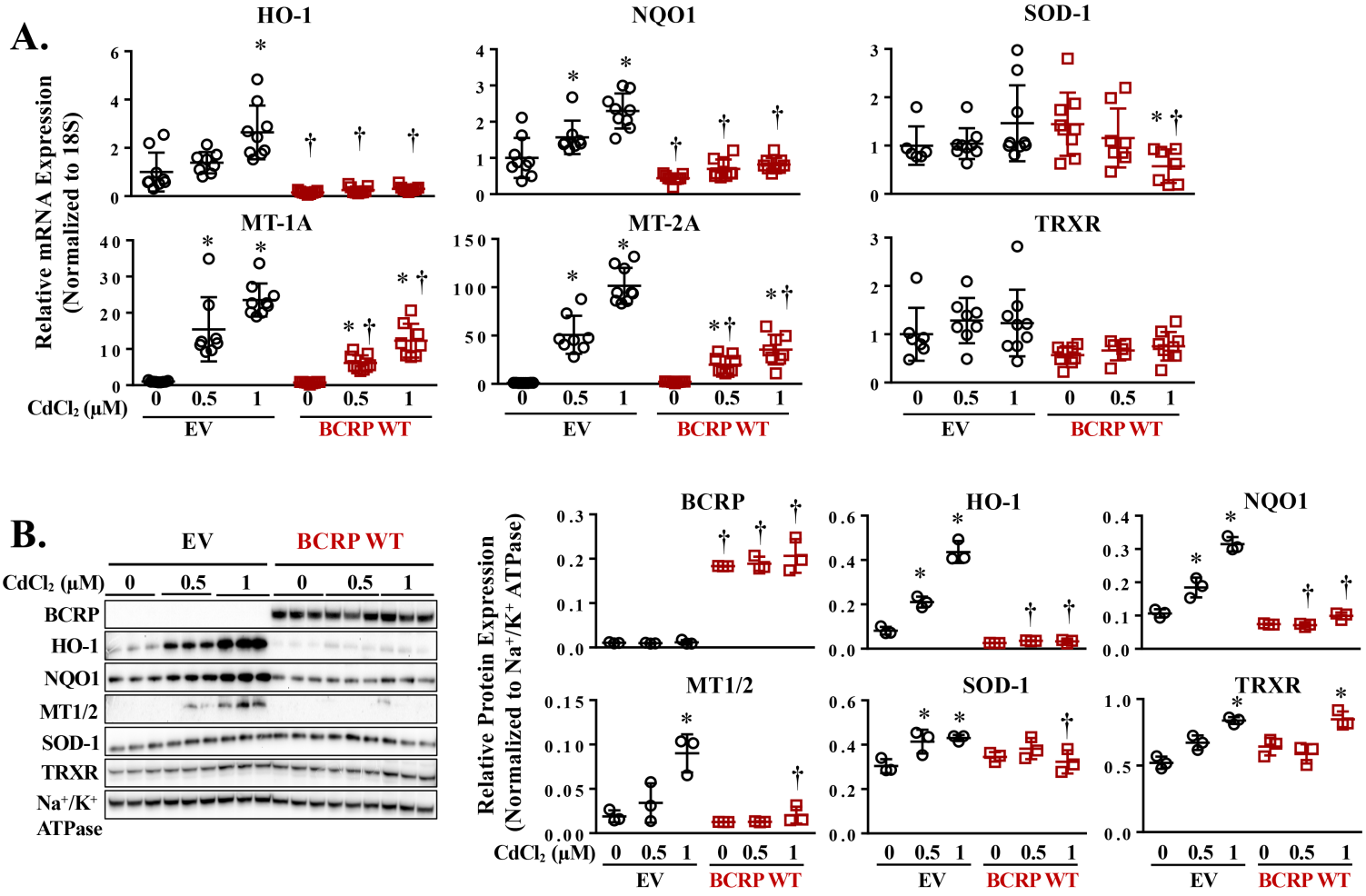

Fig 2

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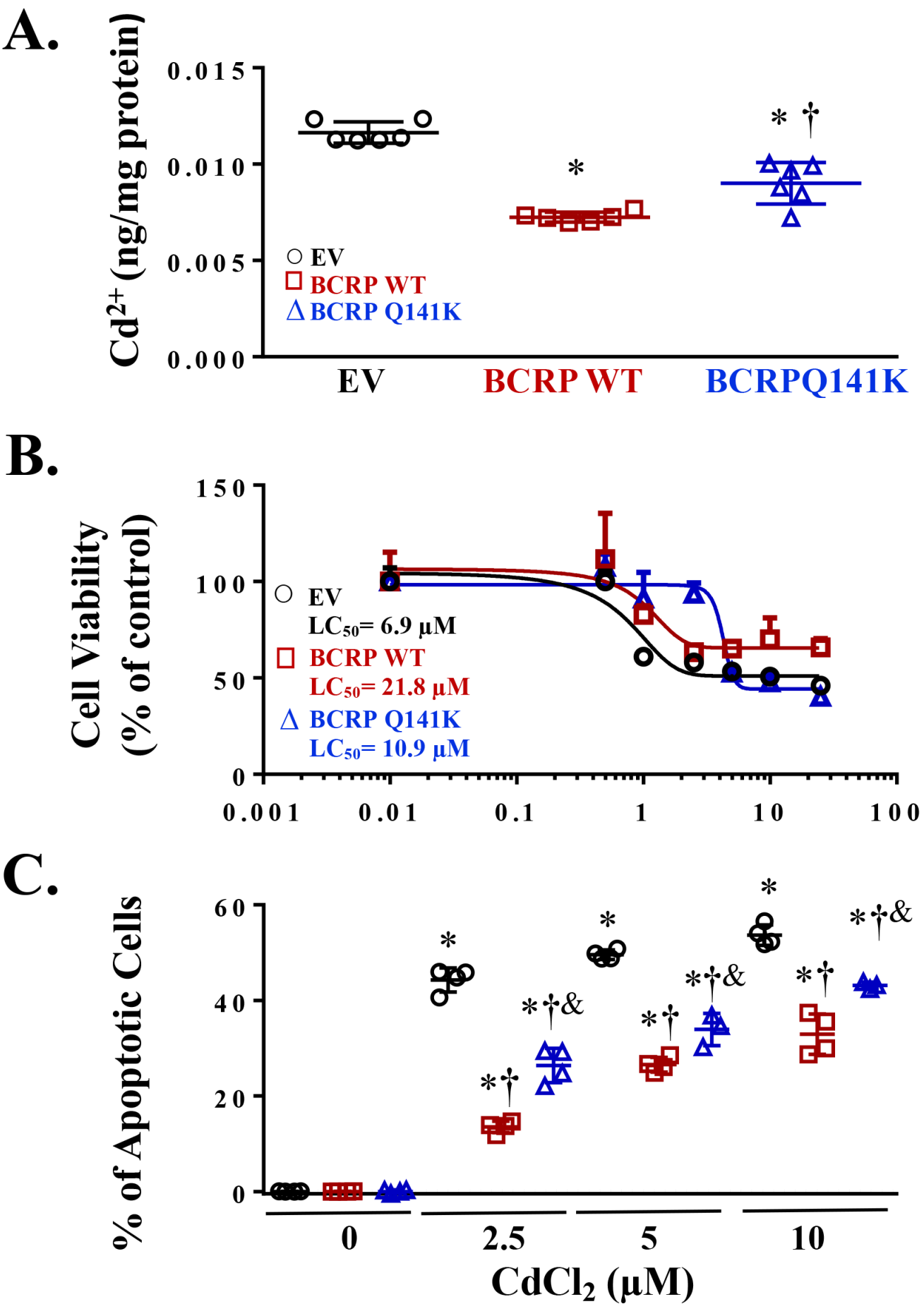

Fig 3

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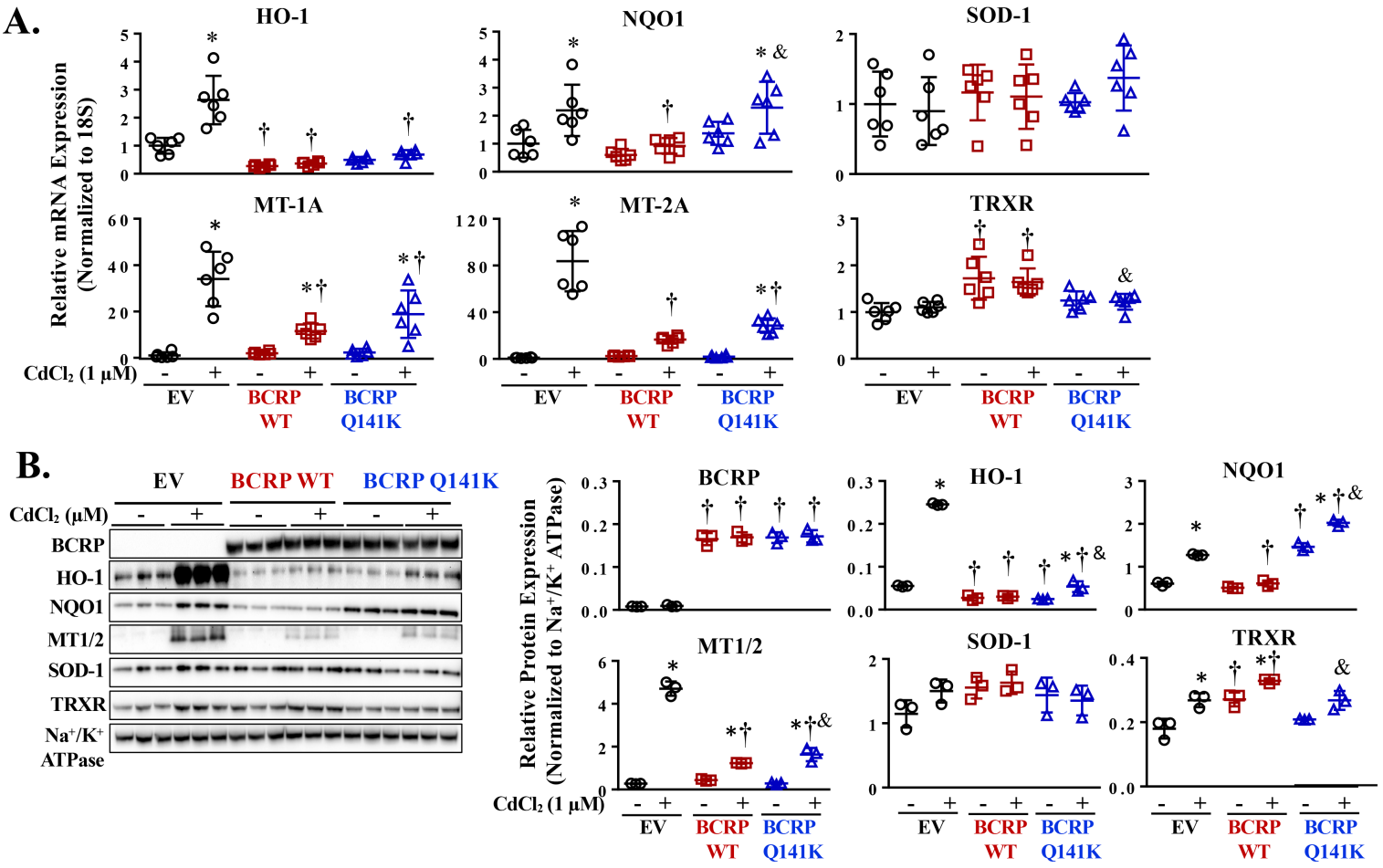

Fig 4

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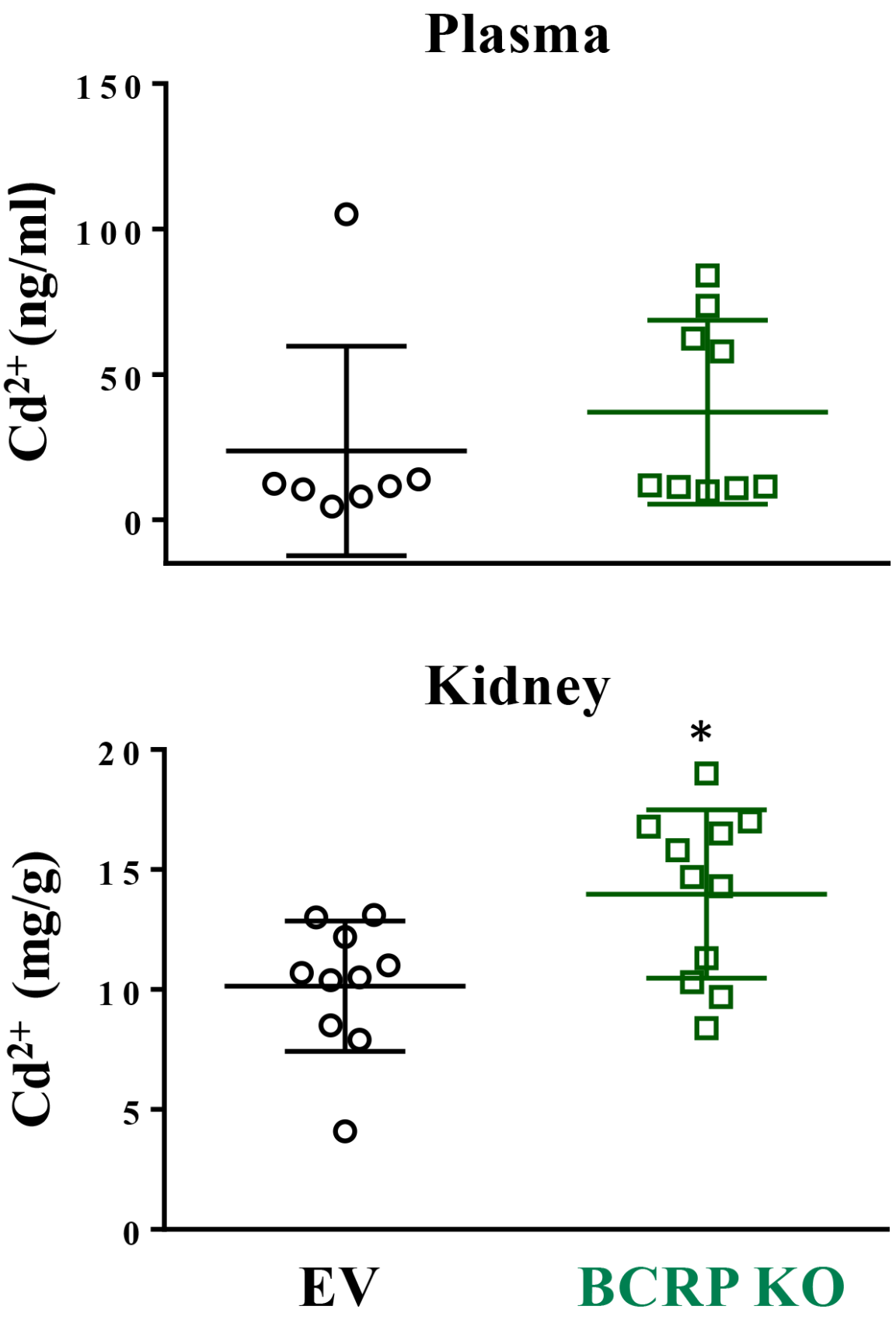

Fig 5