Claudin-3 and Claudin-4 Regulate Sensitivity to Cisplatin by Controlling Expression of the Copper and Cisplatin Influx Transporter CTR1

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ABSTRACT

Claudin-3 (CLDN3) and claudin-4 (CLDN4) are the major structural molecules that form tight junctions (TJs) between epithelial cells. We found that knockdown of the expression of either CLDN3 or CLDN4 produced marked changes in the phenotype of ovarian cancer cells, including an increase in resistance to cisplatin (cDDP). The effect of CLDN3 and CLDN4 on cDDP cytotoxicity, cDDP cellular accumulation, and DNA adduct formation was compared in the CLDN3- and CLDN4-expressing parental human ovarian carcinoma 2008 cells and CLDN3 and CLDN4 knockdown sublines (CLDN3KD and CLDN4KD, respectively). Knockdown of CLDN3 or CLDN4 rendered human ovarian carcinoma 2008 cells resistant to cDDP in both in vitro culture and in vivo xenograft model. The net accumulation of platinum (Pt) and the Pt-DNA adduct levels were reduced in CLDN3KD and CLDN4KD cells. The endogenous mRNA levels of copper influx transporter CTR1 were found to be significantly reduced in the knockdown cells, and exogenous expression of CTR1 restored their sensitivity to cDDP. Reexpression of an shRNAi-resistant CLDN3 or CLDN4 up-regulated CTR1 levels, reversed the cDDP resistance, and enhanced TJ formation in the knockdown cells. Baseline copper (Cu) level, Cu uptake, and Cu cytotoxicity were also reduced in CLDN3KD and CLDN4KD cells. Cu-dependent tyrosinase activity was also markedly reduced in both types of CLDN knockdown cells when incubated with the substrate L-DOPA. These results indicate that CLDN3 and CLDN4 affect sensitivity of the ovarian cancer cells to the cytotoxic effect of cDDP by regulating expression of the Cu transporter CTR1.

Introduction

Cisplatin (cDDP) is an effective first-line therapy for many types of cancer, but the rapid development of resistance during therapy remains a major clinical challenge. cDDP is thought to kill cells predominantly by forming adducts in DNA that block transcription and DNA replication. Mechanisms implicated in cellular resistance include reduced drug uptake, increased drug efflux, increased DNA repair, increased tolerance of DNA damage, and an aberrant apoptosis pathway (reviewed in Stewart, 2007). More recently, the copper (Cu) transporters have been found to modulate the cellular pharmacology of the platinum (Pt) drugs (Howell et al., 2010). Resistance appears to be multifactorial in origin, with no single overarching mechanism predominating even within the same histologic type of tumor. Novel insights into molecular mechanisms are important to the goal of identifying patients whose tumors have a high probability of responding to cDDP and avoiding administration of this drug to patients unlikely to benefit from treatment.

One of the hallmarks of malignant transformation of epithelia is loss of tight junctions (TJs) (Itoh and Bissell, 2003; Mullin, 2004). Disassembly or remodeling of TJs is associated with loss of cell polarity and an increase in motility and invasiveness (Kohler and Zahraoui, 2005; Thiery et al., 2009), and there is an association between the loss of cell-cell adhesion structures and metastasis in many epithelial cancers (Martin et al., 2004). The claudin (CLDN) proteins are essential structural components of TJs. The claudin family consists of 27 members that exhibit distinct developmental stage and tissue-specific expression (Ouban and Ahmed, 2010). Each claudin has four transmembrane domains, two extracellular loops, and a carboxy tail containing a phosphorylation domain and a PDZ protein interaction domain. The proteins form heterodimers or homodimers to produce paired strands connecting adjacent cells, thereby determining the characteristic permeability properties of different epithelial tissues (Sasaki et al., 2003). The patterns of expression of CLDNs in normal tissues, benign tumors, and malignant cancers are complex and appear to be tissue dependent (Hewitt et al., 2006).

Normal ovarian surface cuboidal epithelial cells do not express either CLDN3 or CLDN4, and thus, initial studies...
based on the idea that ovarian cancers arise from this epithelium interpreted the expression of CLDN3 and CLDN4 in ovarian cancers as reflecting up-regulation (Hough et al., 2000; Rangel et al., 2003; Heinzelmann-Schwarz et al., 2004; Santin et al., 2004). However, there is now strong evidence that these tumors arise primarily from the distal fallopian tube, which expresses both of these Claudins (Finch et al., 2006). Recent expression profiling studies have demonstrated a wide range of CLDN3 and CLDN4 expression among epithelial ovarian cancers with a substantial fraction having low level expression (Lee et al., 2007; Levanon et al., 2010; Yoshihara et al., 2010; Karst et al., 2011). Such low expression has been linked to a mesenchymal pattern and poor prognosis in breast (Szasz et al., 2011), esophageal (Lee et al., 2012), colon (Erosz et al., 2011; Matsuoka et al., 2011), and pancreatic (Tsutsumi et al., 2012) cancers. What role these proteins serve in ovarian cancers is largely unknown, and data from studies addressing this question have yielded conflicting results (reviewed in Hewitt et al., 2006; Kominsky, 2006; Ouban and Ahmed, 2010).

CLDN3 and CLDN4 are of particular interest in ovarian cancer therapy because CLDN3 and CLDN4 are the only transmembrane tissue-specific Claudins capable of mediating Clostridium perfringens enterotoxin binding and cytolsis (Katohara et al., 1997); therefore, they have attracted attention as potential therapeutic targets (Morin, 2005; Kominsky, 2006; Kominsky et al., 2007; Yuan et al., 2009; Saeki et al., 2010). We made the novel observation that CLDN3 and CLDN4 modulate sensitivity to the cytotoxic effect of cDDP. We report here that knockdown of CLDN3 or CLDN4 in ovarian cancer cells results in a large increase in resistance to cDDP, which is mediated through down-regulation of Cu influx transporter CTR1 leading to both impaired cDDP uptake and depletion of intracellular Cu.

Materials and Methods

Cell Culture and Transfection. The human ovarian carcinoma cell line 2008 was grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Its two sublines 2008-CLDN3KD-4.5 and 2008-CLDN4KD-5.5, identified here as CLDN3KD and CLDN4KD in which CLDN3 or CLDN4 has been knocked down (Yuan et al., 2009), were cultured in RPMI 1640 medium supplemented with 10% FBS, antibiotics, and 10 μg/ml puromycin. We used pcDNA-hCTR1, a pcDNA3.1 vector containing full-length human CTR1 cDNA and expressing a Genetecin (G418) resistance marker (Moller et al., 2000), to transfect 2008, CLDN3KD, and CLDN4KD cells with Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells transfected with pcDNA3.1 empty vector (EV) served as controls. Transfected cells were selected with 500 μg/ml G418. Surviving clones were combined to create a multiclonal generation.

Drugs and Reagents. A commercial formulation of cDDP was obtained from the University of California—San Diego Moores Cancer Center pharmacy. Doxorubicin, paclitaxel, etoposide, and vincristine were gifts from the San Diego Veterans Affairs Infusion Center Pharmacy. The drugs were diluted to the desired concentrations in RPMI 1640 medium (Thermo Scientific, Logan, UT). The Detergent Compatible Protein kit was purchased from Bio-Rad Laboratories (Hercules, CA) and the tetrazolium compound WST-8 (Cell Counting Kit-8, CCK-8) from Dungo Molecular Technologies (Rockville, MD).

qRT-PCR. CTR1, CTR2, ATP7A, ATP7B or ATOX1 mRNA levels were measured by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). cDNA was generated from mRNA isolated using TRIzol (Invitrogen). Purified mRNA was converted to cDNA using oligo(dT)20 priming and the SuperScript III First-Strand kit (Invitrogen). We performed qRT-PCR on an MyIQ qPCR machine (Bio-Rad Laboratories). The forward and reverse primers for CTR1, CTR2, ATP7A, ATP7B, ATOX1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were, respectively, aggctggcagaaagtcct and gtcgtagctactgaactacgagc, gtcgtagctactgaactacgagc, and gtgcgcatggcatggccctt and gtagctactgaactacgagc. Reactions were prepared using iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer’s recommendations. Samples were prepared in quadruplicate with three independent sample sets being analyzed. The analyses were performed using the Bio-Rad iQ5 system software.

Western Blot Analysis. Cells grown to 80% confluence were removed from the dishes with Cell Dissociation Buffer (enzyme free, PBS-based; Invitrogen, catalog number 13151-014), washed twice with Dulbecco’s phosphate buffered saline (PBS). Whole-cell lysates were prepared in radioimmunoprecipitation assay (RIPA) lysis buffer (mammalian cell lysis kit, MCL-1; Sigma-Aldrich, St. Louis, MA) with protease inhibitors and were centrifuged at 14,000 g for 20 minutes at 4°C. The protein was loaded on SDS-PAGE and separated by electrophoresis. A Bio-Rad Trans-Blot system was used to transfer the proteins to Immobilon-PFL membranes (Millipore, Bedford, MA). Membranes were blocked for 1 hour at room temperature in the Odyssey Blocking Buffer (Li-Cor, Lincoln, NE), followed by incubation overnight at 4°C with specific antibodies. The following primary antibodies were used: rabbit polyclonal anti-mCherry (BioVision, Milpitas, CA), rabbit polyclonal anti-claudin-3 (Invitrogen), mouse monoclonal anti-claudin-4 (Abcam, Cambridge, MA), and mouse monoclonal β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After washing 4 times for 5 minutes each at room temperature in PBS containing 0.1% Tween 20, the blots were incubated for 1 hour at room temperature with fluorescently labeled secondary antibody (Li-Cor, Lincoln, NE) diluted 1:10,000 in the Odyssey Blocking Buffer containing 0.1% Tween 20 and 0.02% SDS. After washes for 5 minutes each in 0.1% Tween 20 PBS and rinse with PBS, the blots probed with fluorescently labeled antibody were imaged using an Odyssey Infrared Imager (Li-Cor).

Measurement of Pt and Cu Accumulation. Whole-cell Pt and Cu content was measured as previously reported elsewhere (Larson et al., 2009). All data presented are the mean of at least three independent experiments, each performed with six cultures per concentration tested. For measurement of Pt in DNA, cells were lysed, and the DNA was harvested using DNAzol (Invitrogen) according to the manufacturer’s protocol. For normalization, DNA was measured before addition of nitric acid using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The DNA samples were then digested in nitric acid and prepared before measurement of Pt by inductively coupled plasma mass spectrometry (ICP-MS), as previously described elsewhere (Larson et al., 2009).

Cell Survival Assay. The 2008, CLDN3KD, and CLDN4KD cells were plated into 96-well plates at a density of 2,000 cells per well and allowed to adhere overnight. The cells were then exposed to increasing concentrations of cDDP or Cu. After 96 hours, the effect of cDDP or Cu on cell survival was determined using the tetrazolium compound WST-8 (Cell Counting Kit-8, CCK-8; Dojindo Molecular Technologies) as described previously elsewhere (Yuan et al., 2011). All experiments were repeated at least three times using three cultures for each drug concentration.

Cloning and Lentivirus Transduction. To rescue CLDN3 and CLDN4 expression in the claudin-knockdown cells, six synonymous point mutations were introduced into shRNA target regions in full-length CLDN3 or CLDN4 cDNA. The shRNA-targeted sequence in CLDN3 was 5′-CAC CGC AAC AAG GAC TAC GTC-3′; the mutations introduced in the shRNA-targeted sequence of CLDN3 were 5′-GAAC AAA GAT TAT GTA-3′. The shRNA-targeted sequence in CLDN4 was introduced in shRNA target regions in full-length CLDN3 or CLDN4 cDNA.
was AAC ATT GTC ACC TCG CAG; the mutations introduced in the shRNA-targeted sequence of CLDN4 were AAT ATC GTA ACT TCA CAA. The shRNA-resistant CDNs were subcloned into pLVX-mCherry vector using In-Fusion cloning strategy (Clontech Laboratories, Mountain View, CA). The silent mutations were sequence verified. For lentivirus production, Lentiv-293T cells (Clontech) were transfected with the CLDN3- or CLDN4-rescuing vector and Lenti-X HTX Packaging Mix containing lentiviral packaging genes using the Xfect transfection reagent, according to the manufacturer’s protocol (Clontech). The produced lentiviruses were used to transduce the CLDN3KD and CLDN4KD cells, and a pool of cells expressing high levels of the fluorescent mCherry tag were isolated by FACS sorting and designated as CLDN3KD-rsc3 and CLDN4KD-rsc4. We used 2008 cells infected with lentiviruses containing a nontargeting scrambled shRNAi and mCherry as control (2008-scb).

**In Vivo Experiments.** All work performed with animals was in accordance with and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California–San Diego. Ten female BALB/c-nu/nu mice, 7 to 8 weeks old (Charles River Laboratories, Wilmington, MA), were inoculated with 2 × 10⁵ 2008-scb, CLDN4KD, or CLDN4KD-rsc4 cells bilaterally into the left and right axillary and flank region. After the tumors were palpable, the mice were treated with a single LD₅₀ dose of cisplatin (10 mg/kg) by the i.p. route. All animals were euthanized when the tumor volume was determined every 2 days by the formula volume = length × width²/2 and plotted as a function of time to generate the in vivo growth curves. When the average tumor size in a group reached 500 mm³, the mice were treated with a single LD₅₀ dose of cisplatin (10 mg/kg) by the i.p. route. All animals were euthanized when the calculated tumor volume reached 1000 mm³ in either of the four groups.

**Measurement of Transepithelial Electric Resistance.** We seeded 100,000 cells in 0.5 ml of complete medium into 12-mm transwell inserts with a membrane pore size of 0.45 μm (Millipore, Carrigtwohill, Ireland) placed in 24-well plate containing 0.75 ml of the growth medium. Transepithelial electric resistance (TER) was measured using an EVOM-Epithelial Voltohmeter (World Precision Instruments, New Haven, CT). Readings were taken 3 days after seeding the cells. The TER values were calculated by subtracting the blank values from the sample values and were normalized to the measured TER values.

**In Situ Tyrosinase Activity Assay.** The 2008, CLDN3KD, and CLDN4KD cells were transiently transfected with the tyrosinase cDNA coexpression plasmid, pCTRY (Bouchard et al., 1989), using FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer’s recommendations. In situ tyrosinase activity was colorimetrically detected in the transfected cells, as previously described elsewhere, by assaying the formation of brown DOPA-chrome that results from the oxidation of colorless L-DOPA (Petris et al., 2000). Cells on coverslips were transfected with the pCTRY expression construct, and 48 hours later they were fixed for 30 seconds in acetone/methanol (1:1) at −20°C. These fixation conditions do not impair the activity of tyrosinase (Toyofuku et al., 1999). Coverslips were then incubated for 1 hour at 37°C in phosphate buffer (0.1 M, pH 6.8) containing 0.15% (w/v) L-DOPA (Sigma-Aldrich) to allow the formation of the brown DOPA-chrome pigment. Cells on coverslips were observed by phase-contrast light microscopy (20x objective). After the images were taken, the cells were solubilized in 0.2 M NaOH, and the total protein concentration was measured using the BCA Protein Assay kit (Pierce, Rockford, IL). Melanin levels were determined spectrophotometrically within 200 μg of each sample by measuring the optical density at 475 nm using a Versamax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA) and presented as the percentage of tyrosinase activity relative to the transfected wild-type 2008 control cells. The values were normalized for variations in transfection efficiency by using the β-galactosidase internal control. All of the transfection results represent the mean of a minimum of three independent transfections assayed in duplicate ± S.E.M.

**Statistical Analysis.** All two-group comparisons used Student’s t test with the assumption of unequal variance. Data are presented as mean ± S.E.M.

**Results**

**CLDN3 and CLDN4 Control Sensitivity of the Ovarian Cancer Cells to cDDP.** We have previously prepared sublines of the human ovarian cancer cell line 2008 in which CLDN3 or CLDN4 was constitutively knocked down by infection with a lentivirus expressing an shRNAi targeting one or another of these two claudins (Yuan et al., 2009). To investigate the role of CLND3 and CLDN4 as regulators of cDDP sensitivity, the cytotoxicity of cDDP to the parental 2008 line was compared with that for the CLDN3 or CLDN4 knockdown sublines (CLDN3KD or CLDN4KD, respectively). CLDN3 mRNA and protein levels were reduced by 88% and 69%, respectively, in the CLDN3KD cells; likewise, CLDN4 mRNA and protein levels were reduced by 64 and 59%, respectively, in the CLDN4KD cells (Yuan et al., 2009). Knockdown of these claudins did not produce any change in the rate of proliferation of the cells in vitro and did not statistically significantly perturb cell cycle phase distribution (data not shown). Figure 1 shows the cDDP concentration-survival curves for each of the cell lines. The IC₅₀ (mean ± SEM) was 0.67 ± 0.13, 2.08 ± 0.24, and 5.36 ± 0.39 μM, respectively, for the parental 2008 cells, CLDN3KD cells, and CLDN4KD cells. Therefore, knockdown of CLDN3 resulted in a 3.1-fold increase (P < 0.01) in resistance to cDDP as compared with the parental 2008 cells. More strikingly, CLDN4KD cells exhibited 8.0-fold (P < 0.001) more resistant to cDDP than the parental cells. These results indicate that both CLDN3 and CLDN4 participate importantly in one or more defense mechanisms that offset the toxicity of this Pt-containing drug.

To determine whether knockdown of CLDN3 or CLDN4 modulated sensitivity to other classes of chemotherapeutic drugs, the parental 2008, CLDN3KD, and CLDN4KD cells were exposed to increasing concentrations of etoposide, vincristine, doxorubicin, and paclitaxel, each of which belongs...
to a different mechanistic class. As shown in Table 1, suppression of CLDN3 or CLDN4 did not statistically significantly alter sensitivity of cells to any of these drugs, indicating that knockdown of either CLDN3 or CLDN4 did not nonspecifically perturb cellular defense mechanisms mediating resistance to these agents.

To determine whether the difference in cDDP sensitivity between the parental and claudin knockdown cells measured in vitro translated into a difference in tumor responsiveness in vivo, the parental 2008, 2008-scb, CLDN4KD, and CLDN4KD-rsc4 cells were xenografted subcutaneously into BALB/c nu/nu mice. The resulting tumors were allowed to grow to an average volume of 500 mm³, and the mice were then treated with a single i.p. injection of an LD₁₀ dose of cDDP (10 mg/kg). Figure 2 shows that the LD₁₀ dose of cDDP produced a much greater response in the parental 2008 or 2008-scb than in the CLDN4KD tumors. The 2008 and 2008-scb tumors shrank in size over the first 6 days after treatment before growth started to resume; however, the equitoxic dose of cDDP did not induce any regression of CLDN4KD tumors of equal size. Notably, the effect of CLDN4 knockdown on the in vivo resistance to cDDP was specific, as reintroduction of an siRNA-resistant CLDN4 construct into the knockdown cells markedly increased the tumor responsiveness to cDDP treatment. Thus, consistent with the effect of CLDN4 on cDDP cytotoxicity in vitro, this claudin is also a major determinant of therapeutic efficacy of cDDP in vivo.

**CLDN3 and CLDN4 Knockdown Decreases cDDP Accumulation.** To determine whether changes in sensitivity to cDDP resulted from changes in cellular Pt accumulation, whole-cell Pt content was measured after a 1-hour exposure to 30 μM cDDP by ICP-MS. As shown in Fig. 3A, the parental 2008 cells accumulated 1.67 ± 0.06 ng Pt/μg S whereas the CLDN3KD and CLDN4KD cells accumulated 1.11 ± 0.02 and 0.82 ± 0.03 ng Pt/μg S, respectively, representing 34% (P < 0.05) and 51% (P < 0.01) reduction in the total accumulation of this Pt-containing drug. Thus, at least part of the observed reduction in drug sensitivity can be accounted for by a decrease in drug accumulation.

Formation of DNA-Pt adducts is believed to be the primary mechanism by which Pt-containing drugs cause cell death. Pt was measured in DNA isolated from cells exposed for 1 hour to 30 μM cDDP (Fig. 3B). The CLDN3KD cells contained 15.9 ± 3.9 Pt/μg DNA, a 15% decrease (P > 0.05) relative to 18.8 ± 3.1 pg Pu/μg DNA in the parental 2008 cells. In contrast, there was 60% reduction of Pt adducts in the DNA of CLDN4KD cells compared with the parental cells (7.6 ± 0.7 versus 18.8 ± 3.1 pg Pt/μg DNA, P < 0.05). Thus, the reduced whole-cell accumulation was accompanied by a proportional decrease in the amount of cDDP reacting with DNA.

**Knockdown of CLDN3 or CLDN4 Decreases the CTR1 mRNA Level.** Given the observation that knockdown of either CLDN3 or CLDN4 rendered cells resistant to cDDP, and the fact that sensitivity of ovarian cancer cells to cDDP is mediated in part by Cu transporters and chaperones (Katano et al., 2003; Holzer et al., 2004b; Samimi et al., 2004; Safaei, 2006; Safaei et al., 2009; Blair et al., 2010; Howell et al., 2010), the relative expression of Cu transporters and chaperones previously shown to be capable of modulating the cellular pharmacokinetics of cDDP was quantified by qRT-PCR (Fig. 4). The mRNA level of CTR1 was 1.8 ± 0.11- (P < 0.05) and 2.4 ± 0.12-fold (P < 0.01) lower in the CLDN3KD and CLDN4KD cells, respectively, relative to the parental 2008 cells, but there was no statistically significant difference in the expression of mRNA for CTR2, ATP7A, ATP7B, or ATOX1 (P > 0.05 for all).

**Rescue of CTR1 Expression Restores Sensitivity to the Cytotoxic Effect of DDP.** To further document that the cDDP accumulation and resistance is mediated through CTR1 in the context of the claudin knockdown cells, we exogenously expressed hCTR1 in the parental 2008, CLDN3KD, and CLDN4KD cells and compared their sensitivities to cDDP. As shown in Fig. 5A, CTR1-transfected 2008, CLDN3KD, and CLDN4KD cells expressed 1.4-, 1.9-, and 3.0-fold higher levels of CTR1 mRNA than their respective empty vector controls. It is noteworthy that the overall levels of CTR1 in the three CTR1 stably transfected cell lines appeared moderate and
similar, indicating that exogenous production of CTR1 is also tightly controlled by cells so as to maintain Cu homeostasis. In concert with the net increase in CTR1 levels, the forced expression of hCTR1 in 2008 cells rendered the cells 1.5-fold (P = 0.05) hypersensitive to cDDP as compared with empty vector control whereas increased expression of CTR1 in the CLDN3KD and CLDN4KD cells produced statistically significant degrees of hypersensitivity to cDDP by a factor of 1.7- (P < 0.05) and 3.5-fold (P < 0.001), respectively (Fig. 5B). This observation is consistent with the concept that knockdown of these claudins reduces cDDP sensitivity through down-regulation of CTR1.

Rescue of CLDN3 or CLDN4 Expression Reverses cDDP Resistance, Up-Regulates CTR1 Levels, and Enhances Tight Junction Formation. To further confirm the selectivity and specificity of the effect of CLDN3 and CLDN4 knockdown with respect to cDDP resistance, TJ formation, and CTR1 expression levels, the expression of these two claudins was restored in the CLDN3KD and CLDN4KD cells by infecting them with a vector expressing CLDN3 and CLDN4 mRNAs that had been mutated to prevent the shRNAi from causing their degradation. To facilitate isolation of rescued sublines, the vector also expressed the red fluorescent protein mCherry. As shown in Fig. 6A, the rescued cells expressed abundant mCherry-CLDN3 and mCherry-CLDN4, but the endogenous CLDN3 and CLDN4 proteins were still suppressed, indicating successful rescue of CLDN3 or CLDN4 expression. Reexpression of CLDN3 and CLDN4 sensitized the CLDN3KD and CLDN4KD cells to cDDP by a factor of 1.2- (P < 0.05) and 2.6-fold (P < 0.001), respectively (Fig. 6B).

Consistent with the cytotoxicity results, rescue of CLDN3 and CLDN4 in the CLDN3KD cells and CLDN4KD cells increased CTR1 mRNA levels from 53 ± 6.4.0 to 84 ± 6.10.2% (P < 0.01) and from 33 ± 4.1 to 78 ± 7.6% (P < 0.001) of that in the parental cells, respectively (Fig. 6C).

The ability of the six cell lines to form TJ was examined by measuring TER. As shown in Fig. 6D, although knockdown or rescue of CLDN3 had little effect on the TJ formation, TER was statistically significantly reduced in the CLDN4KD cells and was rescued to levels similar to those seen in the control cells when infected with the shRNAi-resistant CLDN4 construct. These results clearly demonstrate that restoration of the
expression of CLDN3 and CLDN4 rescued the claudin knockdown phenotypes, thus further supporting the idea that the phenotypic changes are specific to the CLDN3 and CLDN4 knockdown.

**Knockdown of CLDN3 and CLDN4 Perturbs Cu Homeostasis.** CTR1 is the main Cu uptake transporter in human cells, and its down-regulation is associated with reduced Cu accumulation and resistance to its cytotoxic effect (Larson et al., 2010). To determine whether the change in CTR1 expression was sufficient to perturb Cu homeostasis, whole-cell Cu content was measured by ICP-MS in the parental, CLDN3KD, and CLDN4KD cells. As shown in Fig. 7A, the average steady-state Cu level in the 2008 cells was 6.4 ± 1.26 ng Cu/μg S, whereas the CLDN3KD and CLDN4KD cells contained only 4.2 ± 0.87 and 4.0 ± 0.74 ng Cu/μg S. Thus, the knockdown of CLDN3 and CLDN4 resulted in 34.4 (P < 0.01) and 37.5% (P < 0.05) reductions of intracellular Cu levels, respectively. To measure the effect of the expression of these claudins on Cu accumulation, the rate of Cu uptake was determined by measuring the Cu content after exposure of the cells to 100 μM Cu for 1 hour. As shown in Fig. 7B, the net uptake after Cu exposure was 39.2 ± 1.12, 34.8 ± 0.49, and 31.3 ± 1.01 ng Cu/μg S, respectively, in the parental 2008, CLDN3KD, and CLDN4KD cells. Of note, the moderate decrease in Cu uptake was statistically significant for the CLDN3KD (P < 0.05) and CLDN4KD (P < 0.001) cells compared with the parental cells. The reduction in both basal Cu content and the rate of Cu accumulation suggests a possible association of the claudins with Cu homeostasis through regulation of CTR1.

To determine whether the differences in Cu accumulation are attributable to different tolerances for the cytotoxic effect of Cu, the viability of the 2008, CLDN3KD, and CLDN4KD cells was measured during a 96-hour exposure to increasing concentrations of Cu. As shown in Fig. 7C, the CLDN3KD cells were 1.1-fold more resistant to Cu than the parental 2008 cells (IC_{50} 26.3 ± 3.4 vs. 23.7 ± 2.8 μM), but the difference in the IC_{50} was not statistically significant (P > 0.05) although there was a clear decrease in sensitivity of the CLDN3KD cells to the higher Cu concentrations tested. However, the CLDN4KD cells were 1.8-fold (P < 0.05) resistant with an IC_{50} of 42.5 ± 5.4 μM. Consistent with less efficiency in mediating Cu uptake, the CLDN3KD cells demonstrated less susceptibility than the CLDN3KD cells to the growth-inhibitory effect of Cu, indicating that the claudin knockdown was adequate to produce resistance either by down-regulating CTR1 or otherwise limiting the access of Cu to key targets capable of triggering impaired proliferation.

**Effect of CLDN3 and CLDN4 on Tyrosinase Activity.** Cu is crucial for the function of several enzymes and proteins involved in energy metabolism, respiration, and DNA synthesis, notably cytochrome oxidase, superoxide dismutase, ascorbate oxidase, and tyrosinase (Tisato et al., 2010). The activity of tyrosinase was measured as an additional assay of the affect of CLDN3 or CLDN4 knockdown on the ability of Cu to reach downstream targets. Tyrosinase activity can be visualized in situ by the formation of brown-colored DOPA-chrome after transfection with a vector expressing tyrosinase and incubation with L-DOPA. As shown in Fig. 8A, many pigmented cells were observed within the population of 2008 cells, indicating adequate intracellular Cu to support substantial tyrosinase activity. However, in both the CLDN3KD and CLDN4KD cell populations, there was substantially less pigmentation, indicating a marked reduction in availability of Cu to support tyrosinase activity in these cells. Pigmentation was not observed in untransfected cells incubated with L-DOPA (data not shown).

Melanin levels were also measured in the cells shown in Fig. 8B, and the data are presented as the percentage of tyrosinase activity relative to the 2008 cells after correction for variation in transfection efficiency by using a β-galactosidase internal control. The tyrosinase activity in the pcTYR-transfected CLDN3KD cells was 78.9 ± 7.3% (P > 0.05) and in the CLDN4KD cells was 54.5 ± 3.4% (P < 0.05) of that in the pcTYR-transfected 2008 cells. Collectively, these data suggest...
that the loss of CTR1 associated with the claudin knockdown limited the availability of intracellular Cu.

**Discussion**

Pt drugs are a mainstay of treatment of ovarian cancer, and emergence of resistance during therapy is a major therapeutic obstacle. In the case of cDDP, even a 1.5-fold increase in resistance is sufficient to account for clinical failure (Fink et al., 1997, 1998; Lin et al., 1999). We found that knockdown of CLDN3 rendered human ovarian carcinoma 2008 cells 3.1-fold resistant to cDDP whereas knockdown of CLDN4 increased resistance by 8.0-fold. The net accumulation of Pt and the DNA adduct levels were both reduced in the CLDN3KD and CLDN4KD cells. Thus, these claudins have major effects on the cellular pharmacology of cDDP. CTR1 is an influx
transporter for both Cu and cDDP, and our data indicate that knockdown of CLDN3 or CLDN4 reduced CTR1 expression whereas no changes were detected in the expression of any of the other Cu transporters or chaperones. The reduction in CTR1 was sufficient to reduce intracellular Cu and impair the activity of tyrosinase, a key Cu-dependent enzyme. This observation introduces the novel concept that TJ integrity or function controls the expression of CTR1, a key determinant of Pt drug sensitivity in ovarian cancer. Disruption of TJs as a result of reducing the expression of CLDN3 or CLDN4 did not alter sensitivity to four other classes of chemotherapeutic agents, which provides evidence for a selective effect on the mechanisms that control sensitivity to cDDP. This selective effect suggests a potentially clinically exploitable approach to identifying patients likely to be more sensitive to Pt drug treatment.

TJs are no longer regarded as simple permeability barriers, and it is widely accepted that they constitute multifunctional complexes involved in various signaling events. TJs appear to function as suppressors of signaling pathways that control cell–cell adhesion, proliferation, and differentiation. Very little is known about which signal transduction pathways are activated by loss of claudins, or how the claudins link to these pathways. Recent work indicates that ZO-1, one of the other proteins found in TJs, forms a complex (the ZO-1–ZO-3 complex) that interacts functionally and structurally with Crumbs-associated signaling molecules as well as the PAR3–PAR6–aPKC complex (Lemmers et al., 2002; Roh et al., 2002; Hurd et al., 2003). This indicates that different TJ-associated signal transduction systems might influence each other. Functional TJs assemble when epithelial cells reach high cell

Fig. 7. Effect of CLDN3 and CLDN4 expression on Cu accumulation and cytotoxicity. (A) steady-state basal level of Cu in 2008, CLDN3KD, and CLDN4KD cells growing in complete medium. (B) total Cu accumulation after 1-hour exposure to 100 μM Cu. (C) inhibition of cell growth during 96 hours of continuous exposure to increasing concentrations of Cu. (C), 2008; (▲), CLDN3KD; (■), CLDN4KD. Each value represents the mean of four independent experiments, each performed with four to six replicate cultures. Vertical bars, ± S.E.M. *P < 0.05; **P < 0.01; ***P < 0.001.
and Caov-3 ovarian cancer cells sensitized them to cDDP but that the effect was less than 2-fold and the error was large. We have now shown that the levels of CLDN3 and CLDN4 directly control sensitivity to the Pt-containing drug cDDP in 2008 ovarian cancer cells. In pursuit of the mechanism by which the knockdown of these claudins modulates cDDP sensitivity, we found that that knockdown of CLDN3 or CLDN4 resulted in decreased expression of CTR1, and that rescue of the expression of partial CTR1 restored cDDP sensitivity. That resistance to the Pt drug induced by CLDN3 and CLDN4 knockdown is mediated by CTR1 was further supported by the evidence that the CLDN3 or CLDN4 knockdown cells exhibited reduced basal Cu content, impaired Cu uptake, and concomitant resistance to Cu cytotoxicity.

Because cDDP is a polar compound that does not readily cross lipid bilayers, its entry into the cell must be facilitated by membrane transport proteins. The role of CTR1 in this process is well documented, although how CTR1 transports cDDP is not well understood at the molecular level. Knockout of the CTR1 gene, or knockdown of its expression, in mammalian cell lines and deletion of a Ctr1 homolog in yeast cause resistance to cDDP (Ishida et al., 2002; Lin et al., 2002; Larson et al., 2009). Overexpression of a functional CTR1 in an ovarian carcinoma cell line resulted in a moderate increase in intracellular Pt accumulation and the rate of influx (Holzer et al., 2004a). Although it remains possible that knockdown of either CLDN3 or CLDN4 perturbs the expression or function of other processes that import cDDP into the cells, the data presented here provide strong evidence of the conclusion that down-regulation of CTR1 expression plays a central role in the resistance of the CLDN3KD and CLDN4KD cells to cDDP. It is noteworthy that knockdown of either CLDN3 or CLDN4 did not alter the expression of any of the other Cu homeostasis proteins tested and produced no large effects on cell growth or morphology when cultured in vitro, which points toward a specific effect on the expression of CTR1.

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Authorship Contributions
Participated in research design: Lin, Howell.
Conducted experiments: Shang, Lin, Manorek.
Performed data analysis: Shang, Lin, Howell.
Wrote or contributed to the writing of the manuscript: Lin, Howell.

References

**Fig. 8.** Tyrosinase activity in the 2008, CLDN3KD, and CLDN4KD cells transiently transfected with the pcTYR plasmid. (A) in situ activity of tyrosinase visualized by the formation of the brown DOPA-chrome pigment after incubation with L-DOPA substrate. Scale bars, 20 μm. Magnification: 100×. (B) melanin levels estimated spectrometrically for the same transfected cells as shown in A. Melanin was measured in 200 μg of protein lysate and expressed as the percentage of tyrosinase activity relative to the transfected 2008 cells. Vertical bars, ± S.E.M. *P < 0.05. All cells were also transfected with a β-galactosidase reporter gene to control for transfection efficiency.


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