CANCER

Response to comment on "Epigenetic activation of the drug transporter OCT2 sensitizes renal cell carcinoma to oxaliplatin"

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OCT2 plays a key role in synergy between decitabine and oxaliplatin in renal cell carcinoma cell lines.

We identified the organic cation transporter OCT2 repression as an important factor in oxaliplatin resistance to renal cell carcinoma (RCC) (1). The loss of OCT2 expression at both transcription and protein levels was determined in collected paired patient tissues, commercial tissue microarray specimens, and RCC cell lines.

Further epigenetic mechanistic studies revealed that DNA methylation resulted in repressed *OCT2* transcription. A sequential combination therapy demonstrated that epigenetic activation of OCT2 by decitabine (DAC) sensitized RCC cells to oxaliplatin both in vitro and in xenografts.

The first concern raised by Winter *et al.* (2) was whether OCT2 is repressed in the clear cell subtype of RCC (ccRCC) tissues. At the mRNA level, we found decreased expression of *OCT2* mRNA in paired ccRCC tissues, which is supported by both microarray data and reverse transcription quantitative polymerase chain reaction (RT-qPCR) data (1). Because of the variability of *OCT2* mRNA expression among persons, the quantification of gene detection based on a paired design (RCC tissues and matched adjacent nontumor collected from the same patients) is crucial to more accurately determine the differential expression. In our paired RT-qPCR analysis, *OCT2* expression was all down-regulated at various levels in the 38 pairs of ccRCC tissues (Fig. 1). Strong (transcription was reduced by at least 70%, as defined in our paper) and weak repression (transcription was reduced by less than 70%) occurred in 20 and 18 cases, respectively. In addition, Winter *et al.* mentioned that *SLC22A2* was among the top 7% expressed genes in 463 ccRCC cases. Because of the high abundance of *OCT2* in the kidney, it is not surprising that it is ranked in the top 7% of expressed genes even after repression. At the protein level, Winter *et al.* showed different findings for OCT2 expression in ccRCC tissues using their homemade antibody KEK and the commercial MAB6547 from R&D Systems (2). A well-validated antibody is crucial to figure out the difference in protein expression. Because no endogenous OCT2 expression was found in any renal cell lines that we examined, the protein expression patterns in biologically proven positive and negative tissues are important to determine antibody specificity on immunohistochemistry (IHC) application. In addition to its expression in the kidney, OCT2 has also been reported to a lower extent in neurons of the cerebral cortex (3, 4). The antibody we used in our paper is AMAb90791 from Atlas Antibodies. The Human Protein Atlas (HPA) project has mapped OCT2 expression in all major tissues and organs of the human body with this antibody (www.proteinatlas.org/ENSG00000112499-SLC22A2/tissue/primary+data). Citing their data,

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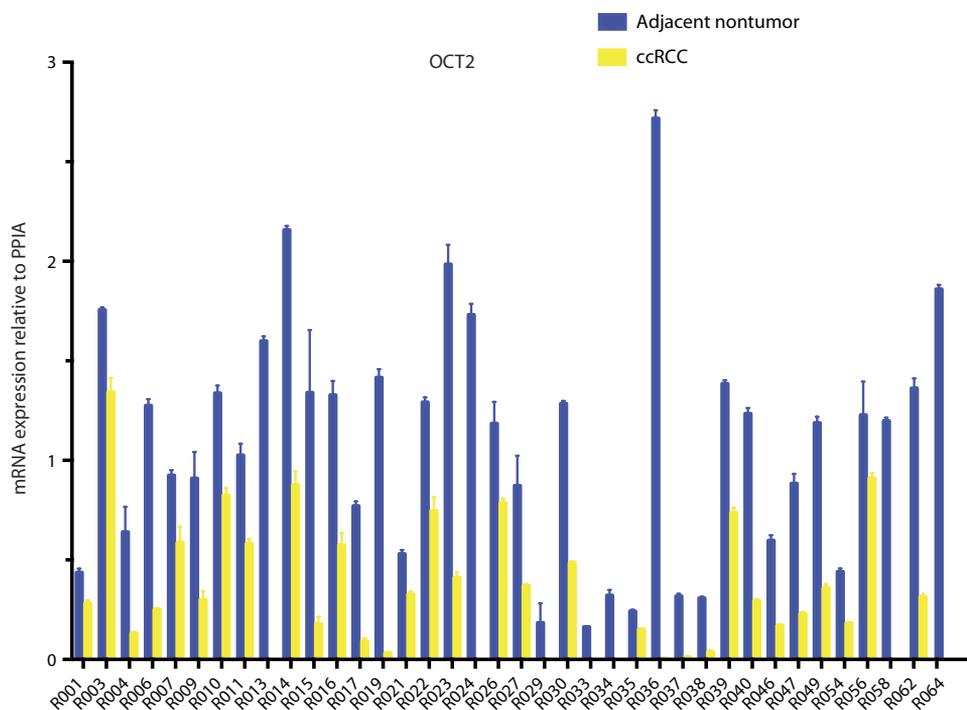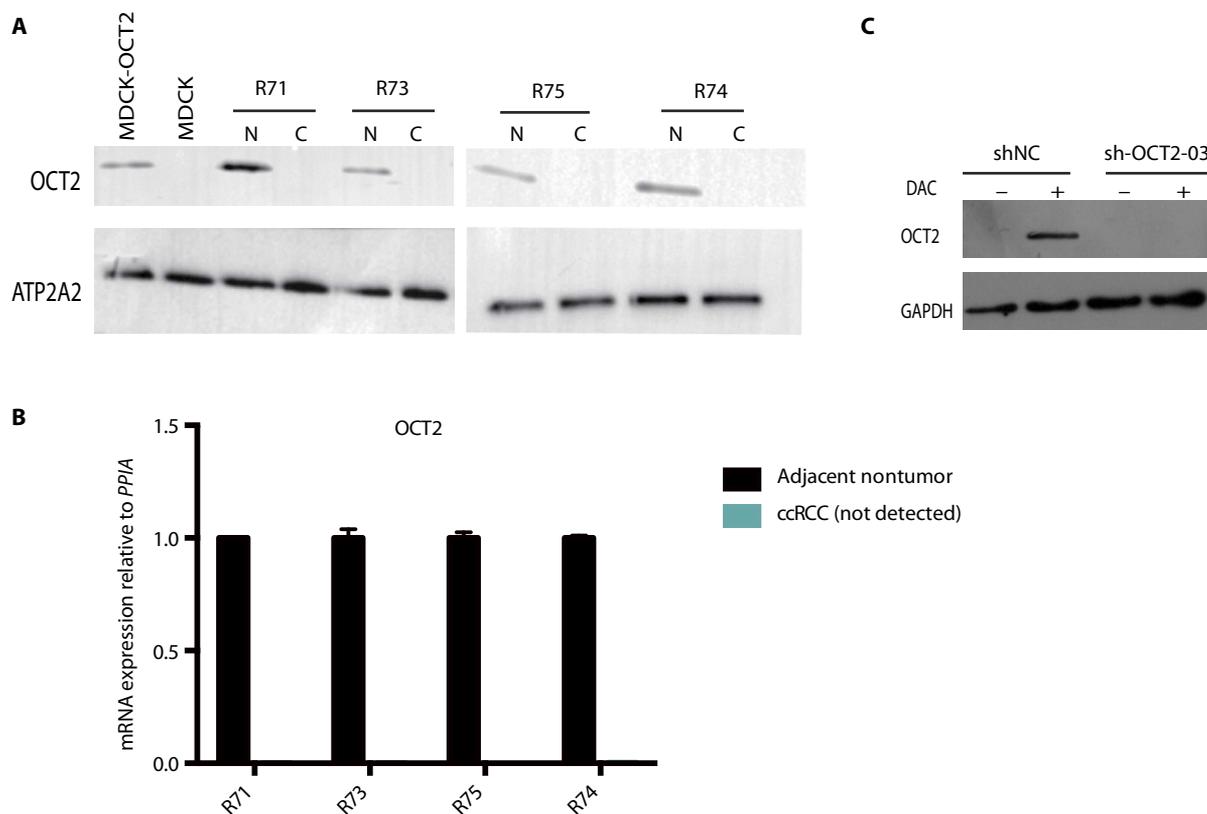
Fig. 1. qPCR analysis of *OCT2* transcription in RCC. RNA was extracted from 38 pairs of RCC and adjacent nontumor tissue samples. Peptidylprolyl isomerase A (*PPIA*) was used as the reference gene to normalize *OCT2* expression in collected tissue samples.

AMAb90791 (labeled as CAB068236 in the HPA database) displayed strong membranous and cytoplasmic positivity in renal tubules, strong cytoplasmic staining in astrocytes of the central nervous system, and mainly negative staining in the remaining normal tissues (representative data are shown in Fig. 2A). Those data are consistent with OCT2 characterization data and RNA expression data. By contrast, in the absence of data in proven negative tissues, it is not easy to determine whether the KEK and MAB6547 antibodies used by Winter *et al.* have been well validated on OCT2 IHC application. In addition, the staining was also detected on the basolateral membrane of tubules using AMAb90791 in our preliminary experiments (Fig. 2B). We regret that we did not carefully check the different results for protein localization because the results from uptake experiments have shown that DAC sensitizes RCC cell lines to oxaliplatin by activating OCT2-mediated uptake of oxaliplatin without boosting its efflux from RCC cells (1). With regard to the localization of OCT2 in renal tubules, OCT2 is known to express predominantly at the basolateral membrane of proximal tubules and to some extent on the apical membrane of distal tubules (3, 4). We have repeated the IHC using two antibodies (MAB6547 and AMAb90791) to validate

the localization of OCT2 (Fig. 2C). Both antibodies showed negative staining in the RCC tumor tissue and basolateral staining in the paired adjacent nontumor tissue. In addition to IHC, Western blot analysis using HPA008567 in the RCC tissues and the paired adjacent nontumor tissues also supported OCT2 repression at the protein level (Fig. 3A) and the mRNA level (Fig. 3B) in RCC. The specificity of this antibody on Western blotting application has been extensively validated in our study (1). Here, we further provide the validation data using OCT2-specific short hairpin RNA (shRNA)–expressed cells (Fig. 3C). Furthermore, Winter *et al.* wrote that both antibodies detected OCT2 protein in their human embryonic kidney (HEK) 293–OCT2 transfectants in immunoblots. However, the specificity and selectivity of KEK to OCT2 are not convincing based on their reported data. In their recent publication, they showed that KEK detected two bands in OCT2-transfected HEK293 cells, and the molecular mass was not shown (5). In their earlier publication, they presented data that KEK detected multiple and smear bands ~80 kDa in OCT2-transfected Madin-Darby canine kidney (MDCK) cells and ~90 kDa in kidney tissues (6). There is a potential glycosylation site in the OCT2 protein sequence, and the apparent larger mass could be caused by glycosylation. However, after deglycosylation treatment, the
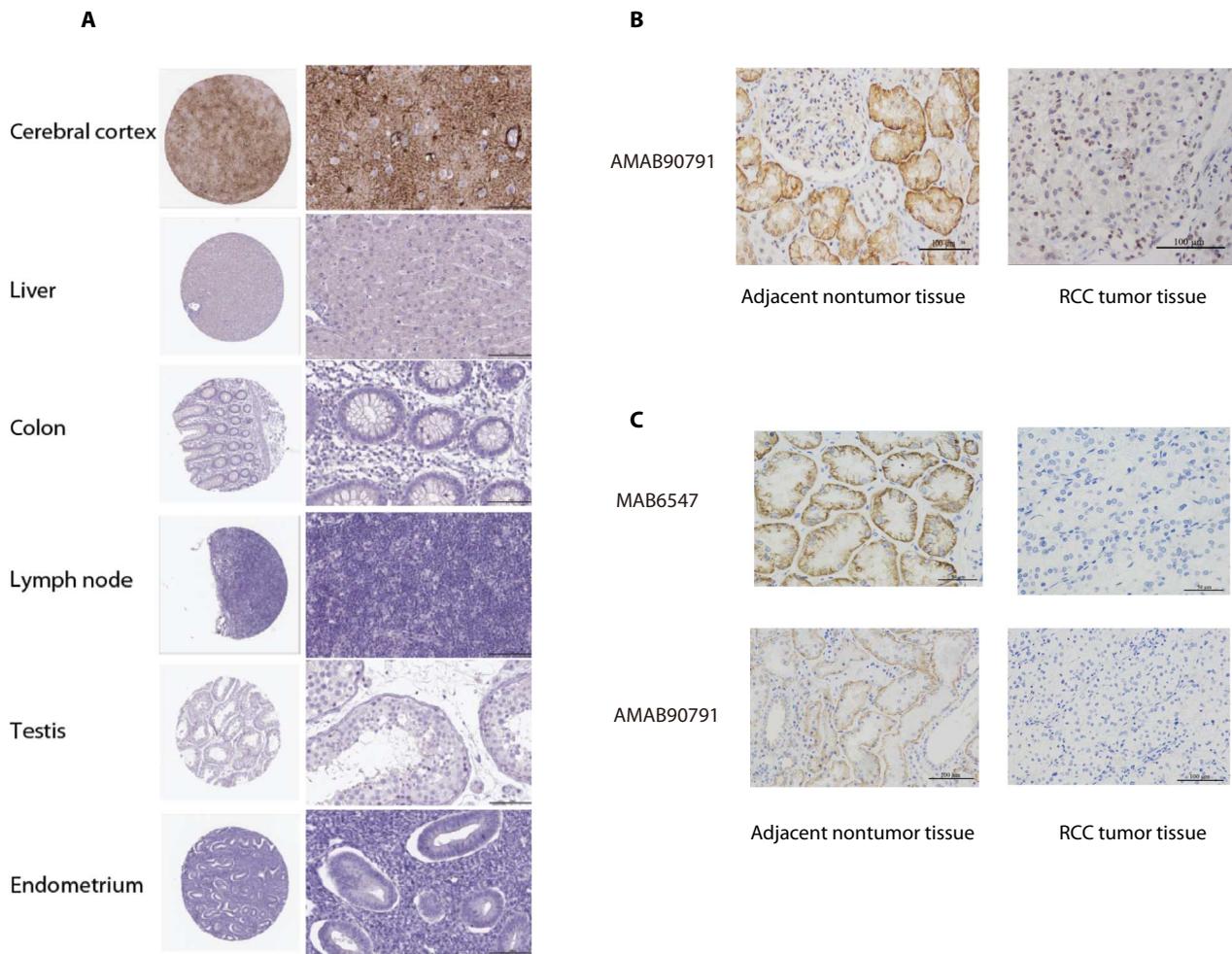
Fig. 2. Representative images of IHC staining for OCT2 protein. (**A**) IHC staining in different tissues using antibody AMAb90791. Data were cited from HPA database. Scale bars, 100 μm. (**B**) Immunostaining of OCT2 in RCC tissues using AMAb90791 antibody. Left, normal kidney tissues; right, RCC tissues. Scale bars, 100 μm. (**C**) Immunostaining for OCT2 using two different antibodies (MAB6547 and AMAb90791) in RCC and adjacent nontumor tissues. Upper image scale bars, 50 μm; lower image scale bars, 100 μm.

Fig. 3. Validation of OCT2 protein expression in RCC. (**A**) Representative images of immunoblots in four pairs of RCC and adjacent nontumor tissues using antibody HPA008567. (**B**) qPCR analysis of *OCT2* transcription in tissues shown in (A). (**C**) Immunoblots using antibody HPA008567 in knockdown cell line lysates from stable ectopic OCT2-expressing cell line. MDCK-OCT2 was used as a positive control to indicate the true signal. RCC cell lines expressing *OCT2*-specific shRNA (shOCT2-03) were treated with 2.5 μM DAC for 72 hours. shNC (negative control) was used as nontargeting shRNA.

authors claimed that the mass was reduced to ~50 kDa, which is below the expected molecular mass of 62.6 kDa. The authors also claimed that preincubation with an antigen peptide completely abolished the detected bands (neither deglycosylation nor peptide blocking data were shown in their paper). However, peptide blocking does not prove selectivity of the antibody because off-target binding activity of the antibody will also be inhibited by preabsorption with the blocking peptide (*7*). Of course, Western blotting cannot be an absolute standardization for antibody binding with the antigen in its native conformation. To verify the sensitivity and membrane localization of the different antibodies, IHC would need to be performed in an independent laboratory and in a larger number of samples for the IHC analysis. Altering the expression patterns of OCT2 by exposure to inducers and repressors of OCT2 during IHC would help the validation.

The next concern of Winter *et al.* was whether drug effects derived from RCC cell lines are of clinical importance. They thought (*2*) that RCC cell lines are of limited clinical importance based on their finding that "DNA methylation and expression of several uptake transporters potentially relevant for novel cancer drugs are altered in RCC cell lines compared to primary tumors and metastases" (*5*). However, because of methylation variability in the population and that each RCC cell line was isolated from an individual patient, the above result does not lead to the conclusion that DNA methylation in ccRCC tissues is at the same level as that in normal tissues. Taking *OCT2* as an example, we had a similar finding as that of Winter *et al.* that DNA methylation levels on *OCT2* in RCC cell lines are higher than those

in RCC tissues. However, with the comparison between RCC and paired adjacent nontumor tissues from the same patients, we found the overall DNA methylation levels on *OCT2* in RCC tissues to be increased, suggesting that hypermethylation on the *OCT2* promoter does occur in RCC tissues (*1*). For the expression of OCT2, we have demonstrated its repression in both RCC tissues and RCC cell lines, as described above. Renal cancer cell lines such as ACHN, 786-O, Caki-1, and Caki-2 are widely used to study the biological effect and relevant mechanism (*5*). Together, we believe that our data derived from RCC cell lines are of clinical benefit for predicting DAC-oxaliplatin combination effects in RCC treatment. To further evaluate the effects of the combination therapy, some work still needs to be carried out. To validate the causality between the decrease of OCT2 and the resistance to the treatment, it is necessary to compare resistance to oxaliplatin between primary tumors and adjacent tissues in the future.

In addition, Winter *et al.*'s publication showed that combination treatment with DAC and cisplatin increases apoptosis in Caki-1 cells compared to nontreated control cells and cells treated with cisplatin alone (*5*). However, on the basis of the data they showed, the drug combination effect is additive between DAC and cisplatin instead of synergistic (% survival in combination ≈ % survival in cisplatin × % survival in DAC). The conclusion that DAC treatment sensitizes Caki-1 cells to cisplatin is unconvincing based on these data. On the other hand, we have used OCT2 knockdown cells to demonstrate that DAC-oxaliplatin synergism depends on OCT2 activation (*1*). Although cisplatin is also a platinum-based compound, the role of

A

B
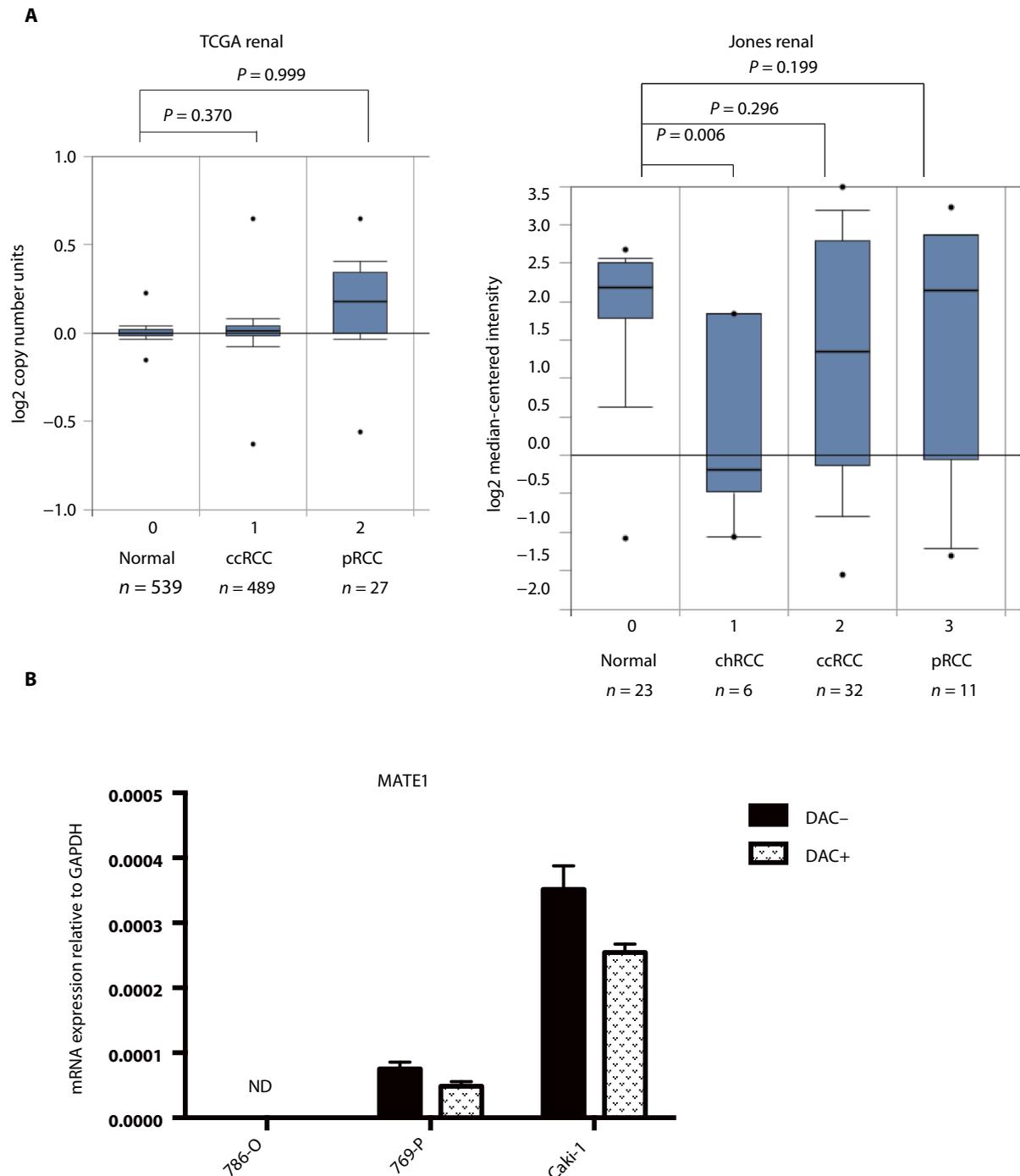
Fig. 4. *MATE1* transcription in RCC. (A) Meta-analysis of *MATE1* transcription in RCC tissues using data sets from the Oncomine database, one-tailed unpaired *t* test (www.oncomine.org/resource/main.html#a%3A6807%3Bd%3A156636562%3Bdso%3AgeneUnderex%3Bdt%3ApredefinedClass%3Bec%3A%5B2%5D%3Bepv%3A150001.151078%2C3508%3Bet%3Aunder%3Bf%3A555154%3Bg%3A55244%3Bp%3A200008889%3Bpg%3A1%3Bpvf%3A3104%2C35361%2C150004%3Bscr%3Adatasets%3Bss%3Aanalysis%3Bv%3A18). **(B)** qPCR analysis of *MATE1* transcription in RCC cell lines. RCC cell lines were treated with DAC (2.5 μM) in RCC cells. ND, not detected.

OCT2 in cisplatin transportation is of debate. Zhang *et al.* reported that OCT2 expression in transfected cells had minor effects on the cellular accumulation and cytotoxicity of cisplatin (*8*). Structure-activity relationship studies further revealed that the nature of amine ligand bound to the platinum is important for the interaction with OCTs. Platinum compounds with two amine ligands such as cisplatin and carboplatin are not favorable substrates of OCTs (*8*). We showed that DAC treatment slightly decreased the median inhibitory concentration of cisplatin in RCC cell lines (*1*). This result supports the dispensable role of OCT2 in the transportation of cisplatin. Winter *et al.* also mentioned the roles of OCT3 and CTR1 in the transportation of oxaliplatin. Both transporters are capable of mediating the cellular

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influx of platinum-based compounds. Compared to OCT2, OCT3 displayed lower abundance in the kidney and lower affinity toward oxaliplatin (9). Another study using CTR1 knockout murine fibroblasts showed that cellular accumulation of oxaliplatin at high concentration (>2 μM) does not depend on CTR1 expression, and loss of CTR1 did not impair cell sensitivity to oxaliplatin, indicating the limited role of CTR1 in mediating cell entry of oxaliplatin (10). These reported data suggest the dispensable role of OCT3 and CTR1 in the oxaliplatin resistance mechanism in RCC. Winter *et al.* also mentioned the efflux transporter protein MATE1 (SLC47A1). MATE1 and MATE2K, which we have discussed in our paper (1), function together at the brush border membrane, mediating the efflux of oxaliplatin into the tubular lumen. Citing data from Oncomine, MATE1 is down-regulated in chromophobe RCC tissues ($P = 0.006$) but not in ccRCC and the papillary subtype of RCC tissues (Fig. 4A). After DAC treatment, *MATE1* expression did not significantly change in RCC (Fig. 4B). Together with our data in *OCT2* knockdown cells and higher-expression cells (1), we could demonstrate the dominant role of OCT2 in DAC/oxaliplatin synergism in RCC cell lines.

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