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RESEARCH PAPER

RAB8 Enhances TMEM205-Mediated Cisplatin Resistance

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ABSTRACT

Purpose To determine whether the small endosomal recycling GTPase, RAB8, plays a role in TMEM205-associated resistance to the chemotherapeutic drug cisplatin.

Methods Antibodies were used as markers for both genes; confocal microscopy was used to visualize their localization in cisplatin-resistant cells. Both single and dual-transfections were performed.

Results Expression of RAB8 was markedly elevated in human cisplatin-resistant cells. We found that TMEM205 was co-localized with RAB8. Dual transfectants with over-expression of both TMEM205 and RAB8 were found to be up to 4-fold more resistant to cisplatin, while cells transfected with RAB8 alone were ~2-fold more resistant.

Conclusions The development of cisplatin resistance appears to be a consequence of pleotropic epigenetic alterations. We unravel the role of one gene, the GTPase RAB8, in this process. Because its highest expression was at an early step of cisplatin resistance, it may be involved in early development of resistance. Increased expression of TMEM205 and RAB8 in double-transfected cells and their increased resistance to cisplatin indicate an additive effect of these two genes, mediating cisplatin resistance. These two proteins are potential biomarkers or targets for gene or chemotherapy.

KEY WORDS cisplatin resistance · RAB8 · TMEM205

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ABBREVIATIONS

DAPI	4′,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
FITC	fluorescein isothiocyanate
GTP	guanosine triphosphate
M6PR	Mannose-6-Phosphate Receptor
miRNA	micro RNA
SDS-PAGE	sodium dodecyl sulfate polyacrylamide *gel* electrophoresis
STX6	syntaxin 6
TGN	Trans Golgi Network

INTRODUCTION

Cisplatin (*cis*-Diamminedichloroplatinum II) was the first platinum compound approved for the treatment of cancer. It revolutionized chemotherapy by improving treatment of a broad spectrum of solid tumors and by facilitating the cure of metastatic testicular germ-cell cancer. However, despite the high efficacy of the compound, the ability of cancer cells to become resistant to the drug remains a significant impediment to successful chemotherapy. Extensive evidence has demonstrated that pleiotropic alterations occur during development of resistance to this metal compound. A variety of genes have been reported to be involved in modulation of resistance, such as DNA damage repair-related genes, nucleotide excision repair (NER) and mismatch repair (MMR) genes (1,2), membrane transporters (3,4), signal transduction and apoptosis genes (5), and cell cycle regulating genes (6). A pleiotropic defect in genes associated with endocytosis and reduced accumulation of drugs and related compounds has been described (7,8). Recently, gene mutations (9) and changes in miRNA

profiles have also been reported (10,11). Comprehensive analyses of a multiplicity of mechanisms involved in cisplatin resistance have been reviewed (12–15).

In a recent study, we reported that a novel transmembrane protein, TMEM205 (16), was associated with cisplatin resistance (17). Analysis of TMEM205 expression profiles in normal human tissues demonstrates higher expression levels in tissues that secrete proteins, such as those of the liver, pancreas, and adrenal gland. Interestingly, although in wild-type cells TMEM205 has been demonstrated to be a membrane protein (17), confocal images revealed that TMEM205 in cisplatin-resistant cells was located in an intracellular compartment at the periphery of the nucleus. Some of the proteins in this region are associated with membrane protein and vesicle trafficking as well as protein secretion (18). The sequence of TMEM205 has led to speculation that it is associated with secretion (19).

In this study, we show that TMEM205 is co-localized with RAB8, which is a small GTPase belonging to the rab family of ras-GTPases and is also a marker of recycling endosomes (20). Members of this family of GTPases are important regulators of intracellular membrane sorting (21). In particular, they are thought to mediate membrane transport specificity (22). A deficiency in the small GTPase RAB8 was shown to inhibit membrane traffic in developing neurons (23). Recently, it was found that a novel RAB8-dependent exocytic traffic pathway is involved in regulation of MT1-MMP proinvasive activity, which is crucial for tumor cell invasiveness (24). To investigate whether the small GTPase RAB8 is also involved in development of cisplatin resistance and whether it augments cisplatin resistance associated with TMEM205, a single gene transfection of RAB8 and a dual transfection of both RAB8 and TMEM205 were conducted. The data presented here indicate that expression levels of RAB8 were elevated in human cisplatin-resistant cells, and overexpression of RAB8 by transfection can increase cisplatin resistance by ~2-fold. The dual transfectants (RAB8 +TMEM205-expressing cells) exhibited up to 4-fold more resistance to cisplatin than vector-transfected cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human epidermoid carcinoma cell line KB-3-1 and its independent cisplatin-resistant derivatives, KB-CP.3 and KB-CP.5, were selected in a single step at 0.3 and 0.5 μg cisplatin/ml, respectively. KB-CP1 and KB-CP20 were selected with stepwise increases to 1 and 20 μg of cisplatin/ml of medium, as described previously (17). All the cisplatin-resistant cells were maintained in the presence of cisplatin, but cisplatin was removed from the medium 3 days prior to preparation of the proteins. All cell lines were grown as monolayer cultures at 37°C in 5% CO_2, using Dulbecco's modified Eagle medium with 4.5 g/l glucose (Invitrogen, Carlsbad, CA), supplemented with L-glutamine, penicillin, streptomycin and 10% fetal bovine serum (BioWhittaker, Walkersville, MD). Cisplatin and other chemicals were purchased from Sigma (St. Louis, MO).

Preparation of Whole Cell Lysates

Cells were lysed in the lysis buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl2, and 0.5% (v/v) Nonidet P-40) and protease inhibitor. The lysates of the cell population were then centrifuged at 1,000 rpm for 5 min. The supernatant was collected, and the proteins were quantitated using BCA reagents as previously described (17).

Immunoblotting and Confocal Analysis

SDS-PAGE immunoblotting was run as recommended by the manufacturer (Invitrogen, Carlsbad CA). Following electrophoresis, the gels were transblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) at 4°C. Immuno-reaction was performed with the desired primary monoclonal antibody directed toward human RAB8 and secondary HRP-conjugated antibodies. Pierce ECL reagents (Pierce Biotechnology, Rockford, IL) were used for developing signals as described by the manufacturer. The primary antibodies, such as Trans Golgi Network (TGN) markers and HRP-labeled secondary antibodies, were purchased from BD Biosciences (San Diego, CA) and Jackson Immuno-Research Lab (West Grove, PA), respectively. For confocal analysis to visualize intracellular localization, cells were cultured in a Lab-Tek Chamber Slide (Nalge Nunc International, Naperville, IL) and fixed with 70% ethanol at −20°C for 15 min or 2.5% Formaldehyde, 0.5%Triton X100, 0.1 mg/ml BSA at room temperature for 15 min, followed by 70% ethanol for 10 min, then reacted with the primary antibodies, followed by reaction with the secondary antibodies labeled with Rhodamine red or FITC or followed by DAPI counterstaining as desired. The primary antibody directed toward TMEM205 was developed in this laboratory (17). For determination of distribution and intensities of immunofluorescent images, cells were monitored under a laser scanning confocal microscope (Zeiss LSM 510 Laser Scanning Confocal Microscope) at 600× magnification.

MitoTracker Mitochondrion Determination

To determine whether TMEM205 is associated with mitochondria, MitoTracker Orange CMTMRos (M7510) (Invitrogen, Carlsbad, CA) was applied in this study. This compound is in an oxidized form and therefore much more stable than the reduced form, CM-H2RMRos (M7512, Invitrogen, CA). The MitoTracker was dissolved in DMSO according to the manufacturer's instructions. Cells were cultured in a Lab-Tek Chamber Slide as described above for 24 h and then washed twice with DMEM without phenol red. The MitoTracker was loaded into the cell chamber at a final concentration of 0.2 mM in DMEM without phenol red, supplemented with FBS and antibiotics. After 30 min incubation at 37°C, cells were washed twice with DMEM without FBS and the color additive and once with PBS, then fixed with 70% ethanol for 15 min at −20°C. Counterstaining with TMEM205 and DAPI, and confocal examination was the same as described above.

Gene Transfection and Assays of Cell Resistance Levels to Cisplatin and Other Compounds

Full-length cDNA for the gene encoding human RAB8 was obtained from OriGene (Rockville, MD), then re-inserted into a mammalian expression vector, pcDNA5.1 (Invitrogen, Carlsbad CA), as described by the manufacturer. Gene transfection was done with Lipofectin (Invitrogen, Carlsbad CA). Stable transfected clones were isolated after selection with hygromycin for RAB8-transfected cells or dual selection with G418 and hygromycin B for double transfection with TMEM205 and RAB8. Cell sensitivities to cisplatin and carboplatin were tested by seeding cells at 5×10^3 in 0.1 ml of medium in a 96-well plate and then counting after 3 days using a Cell Counting Kit (CCK8) as described by the manufacturer (Dojindo, Gaithersburg, MD). Drugs at the

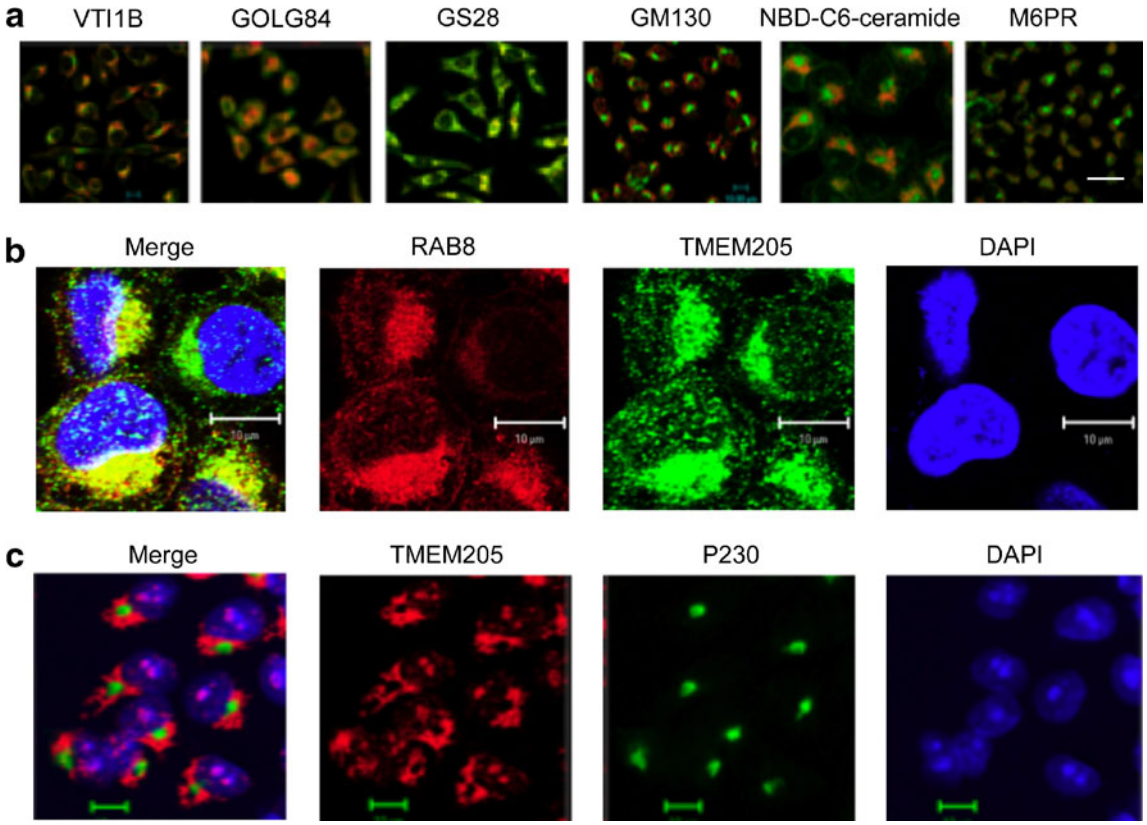

Fig. 1 Colocalization of TMEM205 with RAB8. (**a**) Confocal analysis of the distribution of TMEM205 and TGN-associated proteins in cisplatin-resistant cells. Expression, distribution, and overlapping of TMEM205 (red) and TGN-related proteins (green) are shown for VTI1B, GOLG84, GS28, GM130, NBD-C6-ceramide, and M6PR. Yellowish color represents colocalization, or a very similar distribution of the two proteins. Scale bar: 20 μm. (**b**) Colocalization of RAB8 (red) and TMEM205 (green) in the cisplatin-resistant cells, indicating substantial overlapping of these two proteins near the nucleus (blue). Scale bars: 10 μm. (**c**) Labeling with P230, a TGN marker, shows there is no co-distribution with TMEM205 and serves as a negative control. Images are representative of three independent experiments. Scale bars: 5 μm.

desired concentrations were introduced into each well or dish prior to cell seeding. Control cells were transfected with insert-free vector only. The values are means of triplicate determinations.

RESULTS AND DISCUSSION

Colocalization of TMEM205 With RAB8 But Not VAMP3

Our previous report indicated that the TMEM205 in cisplatin-resistant cells is mainly localized near the periphery of the nucleus. Proteins in this region, including TGN, are known to be involved in the regulation of secretion, vesicle and organelle trafficking, and exocytosis. To determine whether TMEM205 is associated with particular proteins in this region, including those associated with the Golgi network, double staining of TMEM205 with several TGN markers in cisplatin-resistant cells was performed. Figure 1a shows confocal images of markers reported to be in the TGN that were analyzed in this study, including VTI1B, GOLG84, GS28, GM130, NBD-C6-ceramide, and M6PR (Mannose-6-Phosphate Receptor), which is also known as an endosomal marker. As seen, there was no significant overlapping with these proteins. The strongest colocalization was observed between TMEM205 and RAB8, which is a small GTPase, known as a recycling endosomal marker, as seen in Fig. 1b. Rab8, which was stained with red, and TMEM205, stained with green, significantly overlap, as exhibited by the yellowish color in the merged image (Fig. 1b, far left). The DAPI counterstained nuclei show the location of the RAB8 and TMEM205 with respect to the nuclei. In Fig. 1c, triple staining shows that TMEM205 (red) was located in the area surrounding the TGN marker, P230 (green), near the nucleus (blue). No overlap was observed between this TGN protein and TMEM205, indicating that TMEM205 is not co-localized with TGN proteins and that TMEM205 is specifically co-located with RAB8.

We also examined whether TMEM205 was co-located with VAMP3 (24), a protein recycling-related late endosomal marker, but the results showed that there was no obvious correlation (data not shown). This suggests that TMEM205 is found in early, and not late, endosomes.

Increased Expression of RAB8 in Cisplatin-Resistant Cells

As described above, RAB8 was largely colocalized with TMEM205. As RAB8 has been known to be involved in membrane protein trafficking and secretion (25,26), it was then chosen for further investigation. The next question

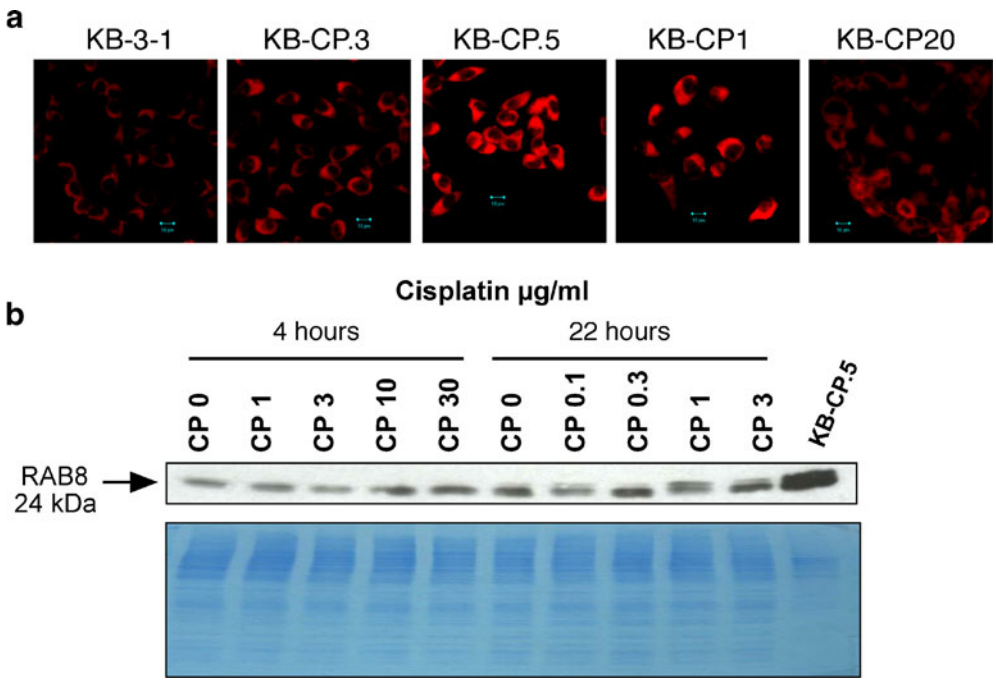

Fig. 2 Overexpression of RAB8. (**a**) Elevated expression of RAB8 seen in cisplatin-resistant cells. KB-3-1 are the wild-type parental cells; KB-CP.3, KB-CP.5, KB-CP1, and KB-CP20 were selected with cisplatin at concentrations of 0.3, 0.5, 1.0, and 20 μg/ml, respectively. Images are representative of three experiments. Scale bars: 10 μm. (**b**) Effect of cisplatin on expression of RAB8 in KB-3-1 cells. Cells were treated with cisplatin at 0, 1, 3, 10, and 30 mg/ml for 4 h, or at 0, 0.1, 0.3, 1, 3 μg/ml for 22 hours. The cisplatin-resistant KB-CP.5 cells served as a positive RAB8 expression control.

that we asked was whether RAB8 also plays a role in the development of cisplatin resistance. A comparison of cisplatin-resistant cells with low to high levels of resistance, KB-CP.3, KB-CP.5, KB-CP1, and KB-CP20, revealed that expression of RAB8 was indeed increased in these cells and reached a peak in KB-CP.5 cells (Fig. 2a). This expression pattern of RAB8 in cisplatin-resistant cells is very similar to that of TMEM205, as reported in a previous paper (17). The highest expression of RAB8 at an early step of cisplatin resistance (KB-CP.5 cells) suggests that RAB8 might also be involved in the early development of cellular resistance to platinum. To determine if it was a primary mechanism or a secondary stress reaction of the cells responding to an exogenous toxic compound, we examined the acute effect of cisplatin on expression of RAB8 in KB-3-1 cells (Fig. 2b). In comparison with the KB-CP.5 cells, there was no significant difference in the expression levels of RAB8 after exposure to cisplatin for 4 h at high concentrations of cisplatin, 1 to 30 μg/ml, or to low concentrations of cisplatin (0.1 to 3 μg/ml) for 22 h, indicating that elevated expression of RAB8 in cisplatin-resistant cells appears as a stable phenotype during development of resistance to the compound.

Rab8 Enhanced TMEM205-Mediated Cisplatin Resistance

To determine whether the elevated RAB8 and TMEM205 seen in cisplatin-resistant cells contribute to cisplatin resistance, we performed a dual transfection (Fig. 3a). Rab8 was inserted into an expression vector, pcDNA5 (Invitrogen, Carlsbad, CA) with a selective marker, hygromycin B, as RAB8/pcDNA5.hyg. As seen in Fig. 3a, transfection of this vector into KB-3-1 cells and TMEM205-expressing cells (KB/TM-neo^r) was followed by a single selection with hygromycin B for RAB8-positive clones and a dual selection with G418 and hygromycin B for TMEM205 and RAB8 positive clones, respectively. Figure 3b shows the formation of colonies after selection with hygromycin B (KB/R) and dual selection of hygromycin B and G418 (KB/TM+R). KB/V is a control, which was transfected with vector only (containing both Hyg and Neo resistance markers), whereas KB is a negative control without transfection of any expression vector, showing no surviving cells after selection. As shown in Fig. 3c, expression of RAB8 was seen at different levels in

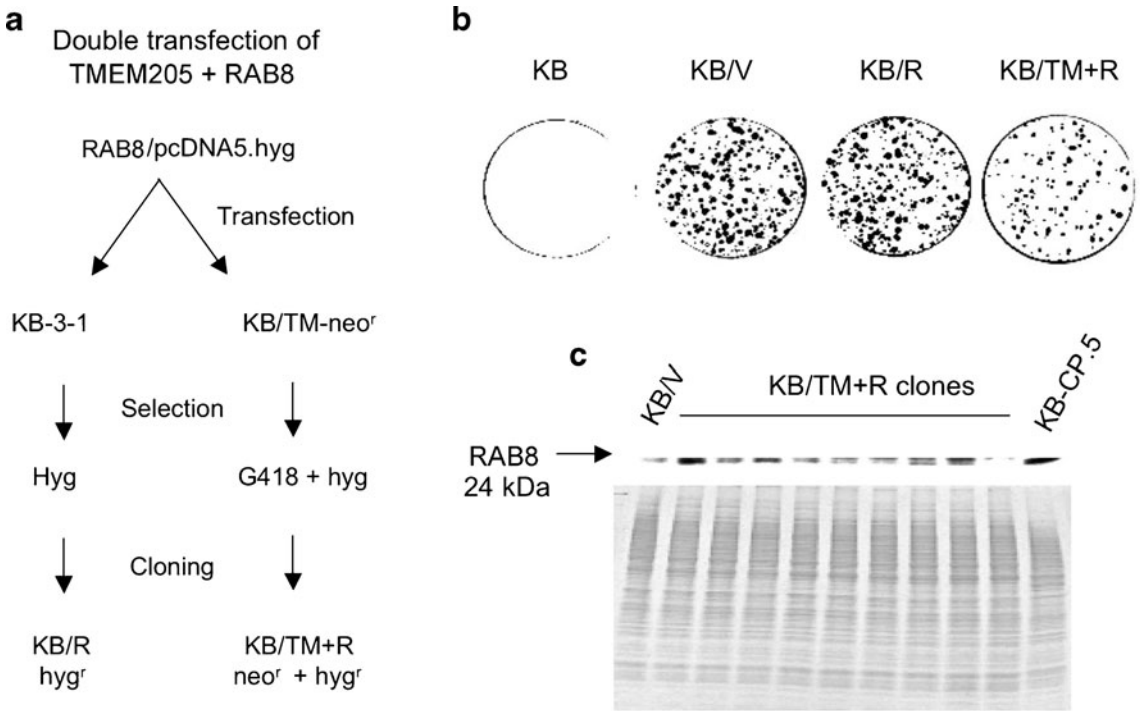

Fig. 3 Dual-transfection of TMEM205 and RAB8. (**a**) Flow diagram showing the double transfection of RAB8 into KB/M-neo^r (TMEM205-expressing cells), then selected with the dual selective markers neomycin and hygromycin B for clones carrying both genes; left column shows a single transfection of RAB8 into the cisplatin-sensitive KB-3-1 cells. (**b**) Colonies formed after transfection and selection with related reagent(s): KB, without transfection of any vector, serving as a negative control; KB/V, transfected with vector only; KB/R, transfected with RAB8; KB/M+R, transfected with TMEM205+RAB8. Images are representative of three independent experiments. (**c**) Immunoblotting showing expression levels of RAB8 in individual doubly transfected clones. KB/V (neo+hyg), which was transfected with a vector carrying both neo and hygromycin markers, but without inserts, served as a negative control. KB-CP.5 served as a positive control for RAB8, and the lower panel shows a Commassie blue-stained gel as a loading control.

the dual-transfected clones (KB/TM+R), while control cells (KB/V) showed weak expression. The KB-CP.5 cells served as a positive control, showing a stronger signal by immunoblotting.

Increased expression of both TMEM205 and RAB8 in double-transfected cells (KB/TM+R) is shown in Fig. 4a, in comparison with the control (KB/V) cells, which show much weaker signals, indicating that the dual transfection was successful. The resistance levels of the double transfectants KB/TM+R to cisplatin and carboplatin, as determined by 3-day killing assays are shown in Fig. 4b, c. As seen in Fig. 4b, KB/TM+R cells are more resistant to cisplatin by ~4-fold, while the TMEM205- (KB/TMEM) or RAB8-transfectants (KB/RAB) are only about 2-fold more resistant than the control KB/V cells. This increased level of resistance to cisplatin indicates an additive effect of these two genes, TMEM205 and RAB8, mediating cisplatin resistance.

Resistance to carboplatin seems not to be enhanced in a double-transfected clone (KB/M+R) (Fig. 4c). This is supported by the fact that overexpression of RAB8 alone (KB/RAB8) did not affect the response of KB cells to carboplatin (Fig. 4c). However, the double-transfected clone (KB/TM+R) still shows about 2-fold more resistance to the compound, mainly due to the effect of TMEM205, as reported in our previous paper (17), where TMEM205 alone produced a similar level of resistance to carboplatin. Why RAB8 affects resistance to cisplatin more than to carboplatin is unclear. Carboplatin is chemically related to cisplatin, but its chemical structure and physical properties are not entirely the same, and they are handled differently by the cell. A similar phenomenon has been observed concerning another platinum drug, oxaliplatin. Oxaliplatin has a complicated pharmacokinetic profile and several mechanisms of action (27). It appears that oxaliplatin-resistant cells often exhibit cross-resistance to cisplatin, but cisplatin-resistant cells exhibit little or no cross resistance to oxaliplatin (28,29).

It has been reported that RAB8 is involved in protein trafficking and secretion (18,25,26,30) and, together with TMEM205, a hypothetical membrane secretory protein, may confer cisplatin resistance by enhancing excretion of cisplatin from cells, though this needs further elucidation.

Cisplatin itself can be found in cells at the periphery of the nuclear region in small vesicles sometimes called platinosomes (31). This localization corresponds to that of TMEM205 and RAB8, shown in this work. This observation suggests that TMEM205 might be involved in

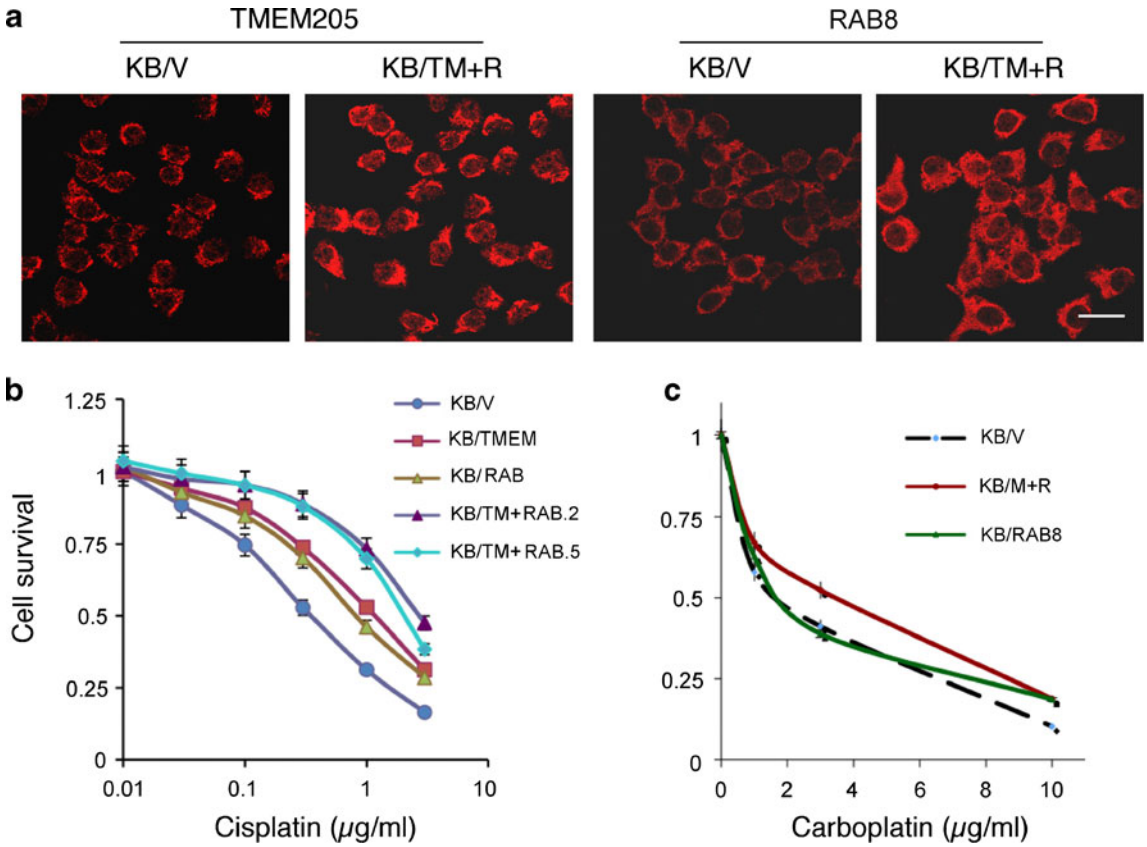

Fig. 4 Rab8 enhances TMEM205-mediated cisplatin resistance. (**a**) Confocal images showing expression of TMEM205 and RAB8 in double transfectants (KB/M+R) in comparison to the recipient cells, KB/V. Images are representative of three independent experiments. (**b**, **c**) Cell survival rates were determined by 3-day CCK8 assays showing levels of resistance of the double transfectants to cisplatin (**b**) and carboplatin (**c**), respectively. The values are means of triplicate determinations. Scale bar: 20 μm.

sequestration of cisplatin. RAB8, known to be involved in exocytic trafficking, could facilitate exocytosis of platinum-containing vesicles from the cytoplasm. Figure 5a shows that syntaxin 6 (STX6) (red) is also significantly co-localized with the TMEM205 (green). Interestingly, the STX6 is seen on the cell surface in the parental KB-3-1 cells, partially overlapping with the TMEM, whereas both STX6 and TMEM205 were co-localized near the nucleus in cisplatin-resistant KB-CP.5 cells. STX6 has been described as a regulator of the protein trafficking machinery involved in membrane trafficking and secretion/exocytosis (32,33). These results suggest that STX6 may also be involved in mediating a secretion/exocytic pathway that leads to reduced accumulation of cisplatin, resulting in cellular resistance to the compound.

To find out whether the TMEM205 might be co-localized with other intracellular organelles, such as mitochondria, MitoTracker Orange CMTMRos (Invitrogen) was employed in this work. Figure 5b shows that the MitoTracker that stained mitochondria (red) was distributed evenly in the cytoplasm, surrounding the nucleus in the cisplatin-sensitive KB-3-1 cells, whereas the mitochondria in the cisplatin-resistant KB-CP.5 cells were located near the nucleus, in a cap-like formation. However, there was little overlap of TMEM205 (green) with MitoTracker in KB-3-1 cells, and they were basically located in different areas in KB-CP.5 cells. These data suggest that there might be no functional correlation of TMEM205 and mitochondria. Nevertheless, additional studies on the role of TMEM205 in combination

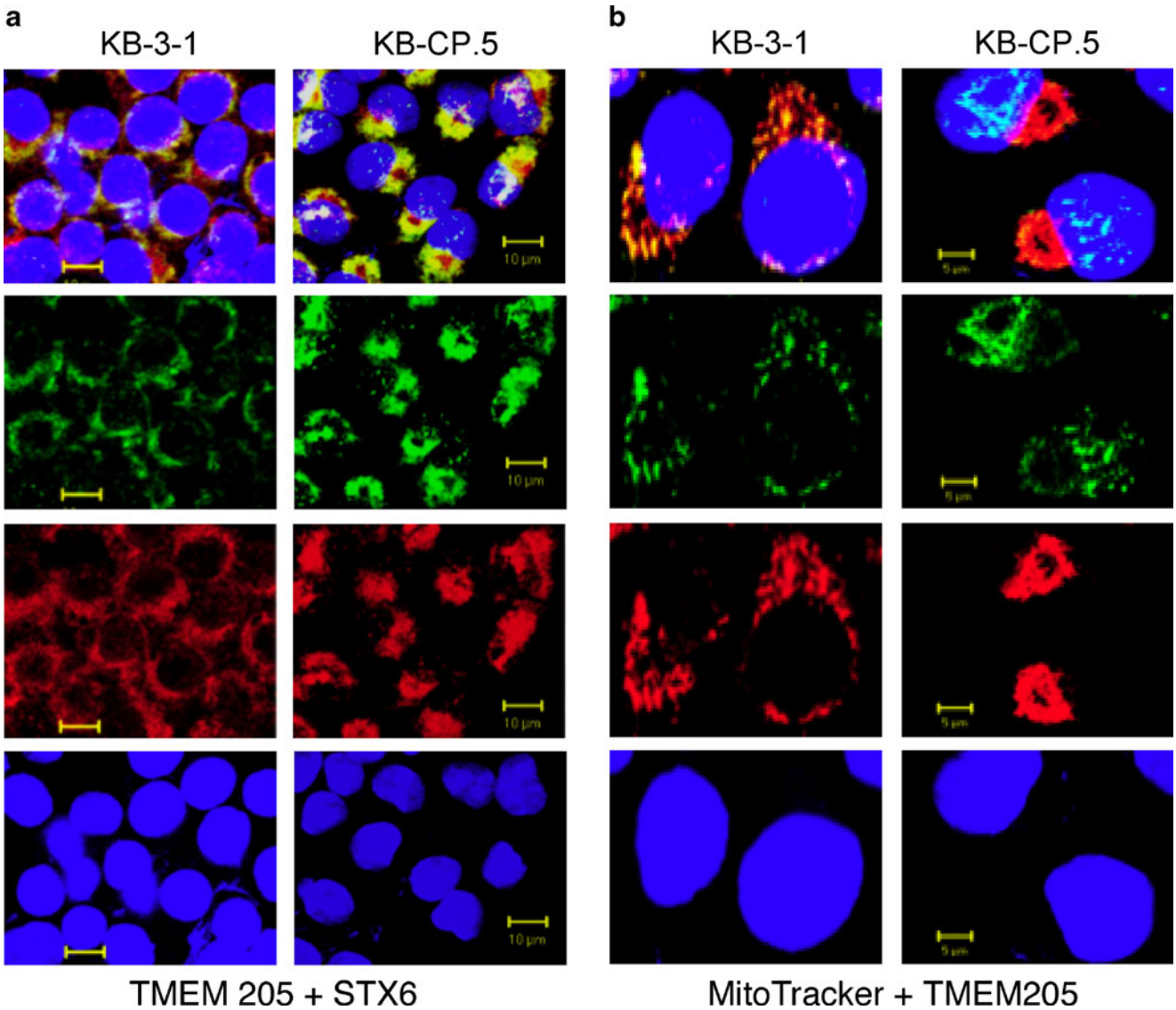

Fig. 5 Colocalization of TMEM205 with STX6, shown by confocal images. (**a**) STX6 red was substantially co-localized with TMEM205, at the peripheral region of the nucleus. The yellowish color indicates overlapping of these two proteins, TMEM205 and STX6. Nuclei were stained with DAPI. Scale bars: 10 μm. (**b**) Little counterstaining of TMEM205 and mitochondria. MitoTracker-stained mitochondria (red) and TMEM205 (green) were located in different areas of the cells. Nuclei were stained with DAPI. Images are representative of at least three independent experiments. Scale bars: 5 μm.

with RAB8, STX6 and other related genes in mediating cisplatin resistance are needed. Taken together, overexpression of RAB8 in human cisplatin-resistant cells, its enhancement of cisplatin resistance, and colocalization of TMEM205 with the functionally related secretion proteins RAB8 and STX6 suggest that a secretion/sequestration pathway may be associated with the development of cisplatin resistance. These proteins are potential targets to be exploited in cancer chemotherapy.

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