TrkB/C-induced HOXC6 activation enhances the ADAM8-mediated metastasis of chemoresistant colon cancer cells

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Abstract. The abnormal expression of tropomyosin receptor kinase (Trk) serves an important role in the promotion of cancer progression. Homeobox C6 (HOXC6) and A disintegrin and metalloproteinase domain-containing 8 (ADAM8) are associated with the invasiveness of cancer cells. However, the exact relationship between these molecules and their downstream signaling pathways in chemoresistant colon cancer cells are largely unknown. Therefore, the current study investigated the association between TrkB/C with HOXC6 and ADAM8 in the induction of drug-resistant colon cancer cell metastasis. The results demonstrated that chemoresistant colon cancer cells exhibited upregulated TrkB/C, HOXC6 and ADAM8 expression. Additionally, but also chemoresistant colon cancer cells demonstrated higher migratory activities compared with parent colon cancer cells. The pharmacological inhibition of TrkB/C activity reduced the phosphorylation of mitogen-activated protein kinase kinase/ERK and subsequently suppressed HOXC6 and ADAM8 expression. In addition, gene silencing of *HOXC6* inhibited ADAM8 and MMP activity, and inhibited the migration and invasion of drug-resistant cancer cells. However, the targeted downregulation of ADAM8 using small interfering RNA failed to suppress TrkB/C-associated ERK-mediated HOXC6 signaling activity. Furthermore, pre-treatment with ADAM10- and ADAM17-specific inhibitors had no effect on attenuating the invasiveness of chemoresistant colon cancer cells. The results indicated that TrkB/C-mediated ERK activation serves an important role in the metastasis of drug-resistant colon cancer cells through the regulation of HOXC6/ADAM8 activity.

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Introduction

Tropomyosin receptor kinases (TrkA, TrkB, and TrkC) encoded by the NTRK1-3 gene family are activated by neurotrophins (1). Trk family proteins were found to be expressed only in cells of the nervous system and identified to be involved in neuronal development and function (2). Various studies have revealed that overexpression of TrkB not only plays a critical role in cancer cell proliferation and metastasis/invasion (3,4) but is also associated with poor clinical outcomes in patients with various cancers (5). The activation of TrkB stimulated by brain-derived neurotrophic factor (BDNF) enhances the resistance of head and neck squamous cell carcinoma to cisplatin through the upregulation of multidrug resistance 1 (MDR1) and X-linked inhibitor of apoptosis protein (XIAP) (6,7). Furthermore, high expression of TrkB and TrkC contributes to tumor proliferation, invasion, and inhibition of apoptosis in colon cancer (8,9). However, the expressional changes, exact roles, and downstream targets of TrkB and TrkC that promote epithelial-mesenchymal transition in drug-resistant colon cancer cells remain unclear.

Human homeobox (HOX) transcription factors regulated by Hox genes play critical roles in embryonic development and differentiation (10,11). Deregulated Hox families (HOXA, HOXB, HOXC and HOXD) have been reported in many cancers, such as breast, ovary, colon, prostate, and lung (10,12,13). HOXC6 is not only overexpressed in numerous cancers but also plays an important role in cancer progression and the survival of cancer cells (14-16). Higher HOXC6 expression is associated with poor prognosis of colon cancer patients and contributes to enhanced cell viability and colony formation of colon cancer cells *in vitro* (17). Furthermore, overexpression of HOXC6 results

in upregulated MDR1 expression through the activation of the promoter activity in colon cancer cells, causing resistance to paclitaxel (18). Although TrkB- or HOXC6-mediated signaling pathways prevent the apoptosis of cancer cells and enhance anticancer drug resistance, the relationship between TrkB/C and HOXC6 in drug-resistant cancer cells is largely unknown.

A disintegrin and metalloproteinase proteins (ADAMs) are tetraspanin-transmembrane proteins that play critical roles in cell adhesion, cell migration, and related signaling pathways (19). In colon cancer cells, ADAM10 induces liver metastasis through the cleavage of endogenous L1-cell adhesion molecule (L1-CAM) (20). We have also reported that ADAM10- or ADAM17-mediated lactate production stimulated by a Toll-like receptor 4 (TLR4) ligand increases the invasiveness and mesenchymal characteristics of colon cancer cells (21). ADAM8 is detected in most cancer tissues, and the serum level of ADAM8 in lung cancer patients is higher than that in the normal group (22). In addition, higher ADAM8 in astrocytoma plays a critical role in tumor cell migration and invasion (23). Furthermore, the upregulation of ADAM8 is associated with brain metastasis of breast cancer through the activation of matrix metalloproteinase 9 (MMP9) (24,25). Although patients with ADAM8-positive colon tumors show a worse prognosis and a lower rate of 5-year disease-free survival (26), no studies have examined the role of ADAM8 in drug-resistant colon cancer cells.

An increase in TrkB or ADAM expression levels triggers various downstream signaling pathways, including the phosphorylation of extracellular-signal-regulated kinase (ERK) (27,28). Furthermore, the upregulation of HOXC6 promotes the proliferation and migration of glioblastoma cells through the activation of mitogen-activated protein kinase (MAPK) (16). Based on these results, we investigated whether TrkB/C- or HOXC6-induced signaling regulates ADAM8 expression to enhance the migratory and invasive abilities of drug-resistant colon cancer cells, which have been established in previous reports (23-26). We also examined whether the TrkB/C- or HOXC6-mediated regulation of ADAM8 levels in drug-resistant colon cancer involves the modulation of ERK activation.

Materials and methods

Cell lines and reagents. Human colorectal carcinoma cell lines HCT116 and HCT8 were obtained from the American Type Culture Collection. These cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Corning Inc.) containing 10% fetal bovine serum (FBS; RMBIO), glutamine, and antibiotics and were incubated at 37˚C in 5% CO_2. Oxaliplatin (Ox) and 5-fluorouracil (5-Fu) were purchased from Sigma-Aldrich; Merck KGaA. The selective tropomyosin receptor kinase (TrK) inhibitor CH7057288 and PD98059 (inhibitor of the MEK/ERK pathway) were purchased from Selleck Chemicals. GI254023X (ADAM10 inhibitor) and Marimastat (ADAM17 inhibitor) were purchased from TOCRIS Biosciences.

Establishment of drug-resistant cell lines. To generate colorectal cancer cell lines with stable chronic resistance to Ox or 5-Fu, HCT116 and HCT8 cells were initially exposed to 1 μM of Ox or 5-Fu in RPMI-1640 medium plus 10% FBS, as previously described with slight modifications (29). When the cells that survived Ox or 5-Fu treatment reached 70-90% confluency, they were subcultured twice a week to confirm their viability. Then, the dose of Ox or 5-Fu was doubled in each surviving population and sequentially increased to 50 μM. All resistant cell lines were maintained and experimented with the presence of 20 μM Ox or 5-Fu in RPMI-1640 medium supplemented with 10% FBS. Finally, the authenticity of the drug-resistant sublines was verified by short tandem repeat profiling according to the ANSI Standard (ASN-0002) from the ATCC Standards Development Organization.

Reverse transcription-quantitative PCR. Total RNA from cells was extracted using an RNeasy Mini Kit (Qiagen), according to the supplier's instructions. cDNA was synthesized from 2 μg of purified total RNA using an Accupower® RT PreMix (Bioneer) and oligo(dT) primer (Bioneer). To evaluate miRNA levels, total RNA was isolated from cells using a miRNeasy Mini Kit (Qiagen). cDNA was synthesized with a Mir-XTM miRNA First-Strand Synthesis Kit (Clontech). The mRNA and miRNA levels were quantified using SYBR-Green (Takara), an ABI7300 real-time PCR system (Applied Biosystems), and specific primer sets (Table I). β-actin was used as an internal control for mRNA expression.

Western blot analysis. Harvested cells were lysed with NP-40 buffer (Elpis Biotech) supplemented with a protease inhibitor cocktail and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA). Protein concentrations were determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce), and equal amounts of proteins (10 μg/sample) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred onto nitrocellulose membranes (Millipore Corp.). The membranes were blocked with 5% non-fat skim milk and probed with primary antibodies. The expression levels of the target proteins were determined using a Chemiluminescence Kit (Advansta Corp.) and an Amersham Imager 600 (GE Healthcare Life Sciences). The following primary antibodies were used: TrkB (#4603), TrkC (#3376), phospho-ERK1/2 (Thr^{202}/Tyr^{204}; #9101), ERK1/2 (#9102), MMP2 (#4022), MMP9 (#3852), E-cadherin (#3195), N-cadherin (#13116), Snail (#3879), and β-actin (#4967) (Cell Signaling Technology); phospho-TrkB (Tyr^{817}; #NBP2-67578), ADAM8 (#NB600-1393), and HOXC4 (#NBP2-56195) (Novus Biologicals); phospho-TrkC (Tyr^{518}; #PA5-40271) (Thermo Fisher Scientific); and HOXC4 (#sc-81965), HOXC6 (#sc-376330), phospho-MEK1/2 (Ser^{218}/Ser^{222}; #sc-7995), and MEK1/2 (#sc-436) (Santa Cruz Biotechnology). The expression levels of β-actin were measured as a control. Densitometry for quantifications of the bands was performed using ImageJ 1.38 software (National Institutes of Health). Relative intensity of bands was calculated by ImageJ and expressed as relative values to β-actin and/or total protein.

Small interfering RNA (siRNA) or micro RNA (miRNA) transfection. Human ADAM8-siRNA (5'-GCA TCA TCG TCT ACC GCA ATT-3'), HOXC6-siRNA (5'-CUC GUU CUC GGC UUG UCU A-3'), and negative control siRNA (cat. no. SN-1001-CFG) were obtained from Bioneer. Transfection

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Target	Primers (5'-3')	
	Forward	Reverse
TrkA	AACCTCACCATCGTGAAGAGT	TGAAGGAGAGATTCAGGCGAC
TrkB	ACCCGAAACAAACTGACGAGT	AGCATGTAAATGGATTGCCCA
TrkC	GCCAGTATCAACATCACGGAC	AGCCGGTTACTTGACAGGTTT
HOXC4	GAGCGCCAGTATAGCTGCAC	GCGACTGTGATTTCTCGGGG
HOXC6	ACAGACCTCAATCGCTCAGGA	AGGGGTAAATCTGGATACTGGC
ADAM8	GAGGGTGAGCTACGTCCTTG	CAGCCGTATAGGTCTCTGTGT
ADAM10	ATGGGAGGTCAGTATGGGAATC	ACTGCTCTTTTGGCACGCT
ADAM12	AACCTCGCTGCAAAGAATGTG	CTCTGAAACTCTCGGTTGTCTG
ADAM17	GACTCTAGGGTTCTAGCCCAC	GGAGACTGCAAACGTGAAACAT
β-actin	ATCCACGAAACTACCTTCAA	ATCCACACGGAGTACTTGC

Trk, tropomyosin receptor kinase; HOX, Homeobox; ADAM, A disintegrin and metalloproteinase domain-containing 8.

with 200 nM siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was performed according to the supplier's instructions. The cells were used for subsequent experiments at 48 h after transfection.

Cell viability assay with Cell Counting Kit-8 (CCK-8). The viability of the cell treated with Trk inhibitor or transfected with siRNA was measured using a CCK-8 (Enzo Life Sciences) according to the supplier's protocol. Briefly, the chemoresistant cells, which were pre-treated with 2 μM CH7057288 or 200 nM HOXC6-siRNA, were seeded into 96-well plates ($2x10^4$ cells/well) and then incubated for 24 h. For comparison, control cells exposed to DMSO or control siRNA were cultured with same media. After treatment, the cells were stained with 10 μl of CCK-8 dye in 90 μl of culture medium for 2 h at 37˚C. The absorbance was measured at 450 nm.

Migration and invasion assays. According to the supplier's instructions, the migratory and invasive activities of cancer cells were determined using a CytoSelect™ Tumor Transendothelial Migration Assay Kit (Cell Biolabs, Inc.) and CultreCoat 96-well Medium BME Cell Invasion Assay Kit (R&D Systems), respectively. The relative fluorescence units (RFUs) of migrated cells were determined by a microplate reader. Briefly, drug-resistant colon cancer cells ($5x10^5$/well) were incubated for 48 h to form a monolayer on the upper surface of the membrane inside the insert and then stained with cell tracking solution (CytoTracker™) to detect migration. After culture for 24 h, fluorescence of lysate derived from cells inside insert were measured with a fluorescence plate reader at 480/520 nm (Perkin Elmer Wallac 1420 VICTOR™; Perkin Elmer). The relative invasion of cells was also compared with the fluorescence intensity of calcein-AM stained invading cells measured by a microplate reader (Perkin Elmer Wallac 1420 VICTOR™). Harvested migrating cells were suspended in calcein-AM/cell dissociation solution and then stock solution was diluted serially. Fluorescence of cell lysates was read at 480/520 nm. Relative invasiveness of chemoresistant colon cancer cells was analyzed using standard curve.

Measurement of gelatinase activity. The gelatinase activity of colon cancer cells was detected using the Gelatinase (Gelatin Zymography) Assay Kit (BioVision, Inc.), according to the supplier's instructions. Briefly, fresh cells ($2x10^6$/sample) were homogenized with cell lysis buffer and incubated for 5 min on ice. The supernatants collected by centrifugation were used to measure the amount of protein using a BCA Protein Assay Kit (Pierce), mixed with a gelatinase substrate, and then the fluorescence at Ex/Em 490/520 nm was measured.

Statistical analysis. Student's t-test and one-way analysis of variance using SPSS version 24.0 statistical software (IBM Corp.) were used for all statistical analyses. Bonferroni post hoc analysis was performed following ANOVA for multiple comparisons. Data are presented as the mean ± standard deviation (SD). Differences were determined to be statistically significant at $P<0.05$ and highly significant at $P<0.005$, respectively.

Results

The expression levels of phosphorylated TrkB/C, HOXC6 and ADAM8 are significantly increased in chemoresistant colon cancer cells. The expression of TrkB in colon cancer is higher than the level in normal tissue (8), and the activation of Trk receptors triggers various downstream signaling pathways, including MEK/ERK (27). Based on previous reports, we first investigated whether the Trk family proteins are overexpressed, resulting in MEK/ERK activation in drug-resistant colon cancer cells. In Ox-resistant (OxR) and 5-Fu-resistant (FuR) colon cancer cells (HCT116_OxR, HCT116_FuR, HCT8_OxR and HCT8_FuR), the mRNA expression of TrkB and TrkC but not TrkA was enhanced than that of parent cancer cells (Fig. 1A). Immunoblotting assays also revealed that the phosphorylation of TrkB/C or activation

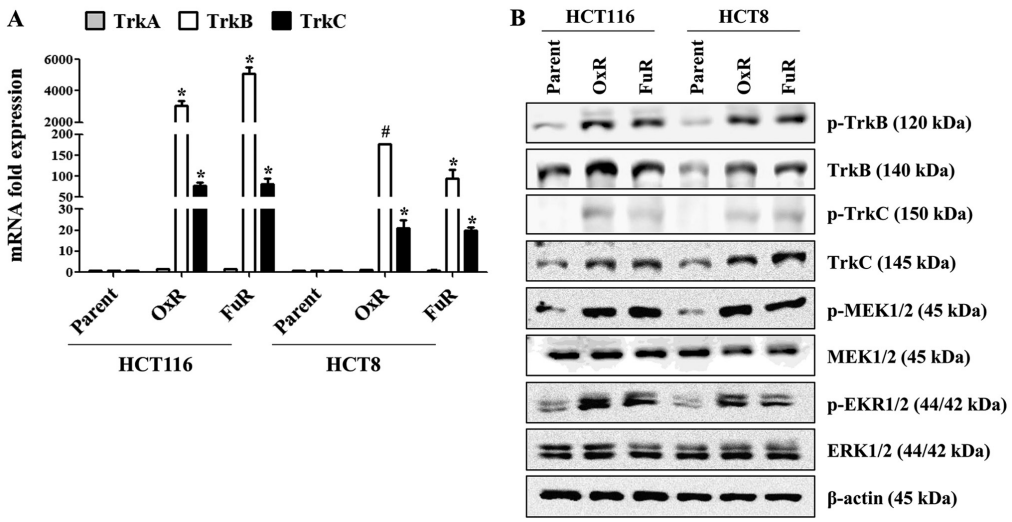

Figure 1. Expression of phosphorylated TrkB and TrkC is significantly increased in chemoresistant colon cancer cells. OxR or FuR colon cancer cells and parent cells were seeded into 6-well plates ($1.5x10^5$/well) and cultured for 24 h. (A) Reverse transcription-quantitative PCR was performed to determine the relative expression of TrkA, TrkB and TrkC. *P<0.05 and #P<0.005 vs. resistant cells. (B) The total protein of each group was subjected to western blot analysis with the indicated antibodies. β-actin served as an internal control. The results are representative of three independent experiments. Trk, tropomyosin receptor kinase; OxR, oxaliplatin resistant; FuR, 5-Fu-resistant.

of MEK and ERK in chemoresistant colon cancer cells was higher than that of the parent cancer cells (Figs. 1B and S1). We also examined whether HOXC6 and ADAM8 expression are upregulated in drug-resistant colon cancer cells. Although the mRNA expression of HOXC4 was prominently increased in HCT116_OxR and HCT8_OxR cells (Fig. 2A) and HOXC6 mRNA and protein expression were markedly increased in all chemoresistant colon cancer cell lines, the slightly upregulated HOXC4 protein was detected using two different antibodies only in HCT8_FuR cells (Fig. 2B). Among the upregulated ADAM family proteins, ADAM8 mRNA expression was significantly higher than other ADAM family members (Fig. 2C). Chemoresistant colon cancer cells upregulated the expression of ADAM8, MMP2 and MMP9 and mesenchymal markers, N-cadherin and Snail, whereas the expression of E-cadherin was downregulated (Fig. 2D). The migratory or invasive activity of HCT116_OxR, HCT116_FuR, HCT8_OxR and HCT8_FuR cells was significantly enhanced (Fig. 2E), and the potential contribution of MMP2 and MMP9 to the metastatic activity was detected through gelatinase activity assays (Fig. 2F). These results suggest that the expression levels of TrkB/C, HOXC6 and ADAM8, which are well-known regulators of cell migration, might influence drug-resistant colon cancer cell metastasis.

TrkB/C-mediated ERK activation regulates HOXC6 and ADAM8 activity during the metastasis of chemoresistant colon cancer cells. We next investigated whether the activation of TrkB/C and related downstream target molecules control the migration of drug-resistant colon cancer cells. After confirming the concentration of Trk inhibitor used in this study had no influence on cell survival and proliferation (Fig. S2A and B), we examined the role of TrkB/C-mediated signaling pathways in the promoting of HOXC6 and ADAM8 expression. Pharmacological inhibition of Trk using a selective TrK inhibitor (CH7057288) prominently suppressed the expression of both total and phosphorylated TrkB/C (Figs. 3A and S3), phosphorylation of MEK/ERK (Fig. 3B), expression of HOXC6 (Fig. 3B), and level of ADAM8 (Fig. 3B). Pretreatment with CH7057288 effectively prevented the migratory and invasive activity of chemoresistant colon cancer cells and the action of gelatinase by inhibiting MMP (Fig. 3C and D). Specific inhibition of ERK (Fig. 4A), which is a downstream target of TrkB/C, using PD98059 reduced the expression of HOXC6 and ADAM8 and the induction of mesenchymal markers in chemoresistant colon cancer cells, whereas treatment with PD98059 failed to attenuate the expression of phosphorylated TrkB and TrkC (Fig. 4B). Furthermore, PD98059 treatment significantly prevented the migration and invasion of drug-resistant colon cancer cells through the inhibition of MMP activation (Fig. 4C and D). These results suggest that TrkB/C-induced ERK signaling plays an essential role in the activation of HOXC6 and ADAM8 during the metastasis of chemoresistant colon cancer cells.

A HOXC6-mediated ADAM8 activation cascade regulates the metastasis of drug-resistant colon cancer cells. We finally investigated the ability of HOXC6 and ADAM8 to control the migratory activity of drug-resistant colon cancer cells. We first confirmed that targeted downregulation of *HOXC6* using siRNA had no role in the viability of drug-resistant colon cancer cells (Fig. S2A and B). Gene silencing of *HOXC6* with siRNA efficiently inhibited the expression of ADAM8, MMP2, and MMP9 and the upregulation of N-cadherin and snail, but did not influence the activation of the TrkB-mediated ERK signaling pathway (Fig. 5A). In addition, targeted inhibition of HOXC6 prominently suppressed the migration, invasion, and gelatinase activity of chemoresistant colon cancer cells (Fig. 5B and C). Although the downregulation of ADAM8 with siRNA failed to reduce the expression level of TrkB/C and related downstream signaling molecules, such as ERK and HOXC6 (Fig. 6A and B), the metastatic activity of chemoresistant colon cancer cells was significantly decreased by suppressing the action of MMP (Fig. 6C and D). However,

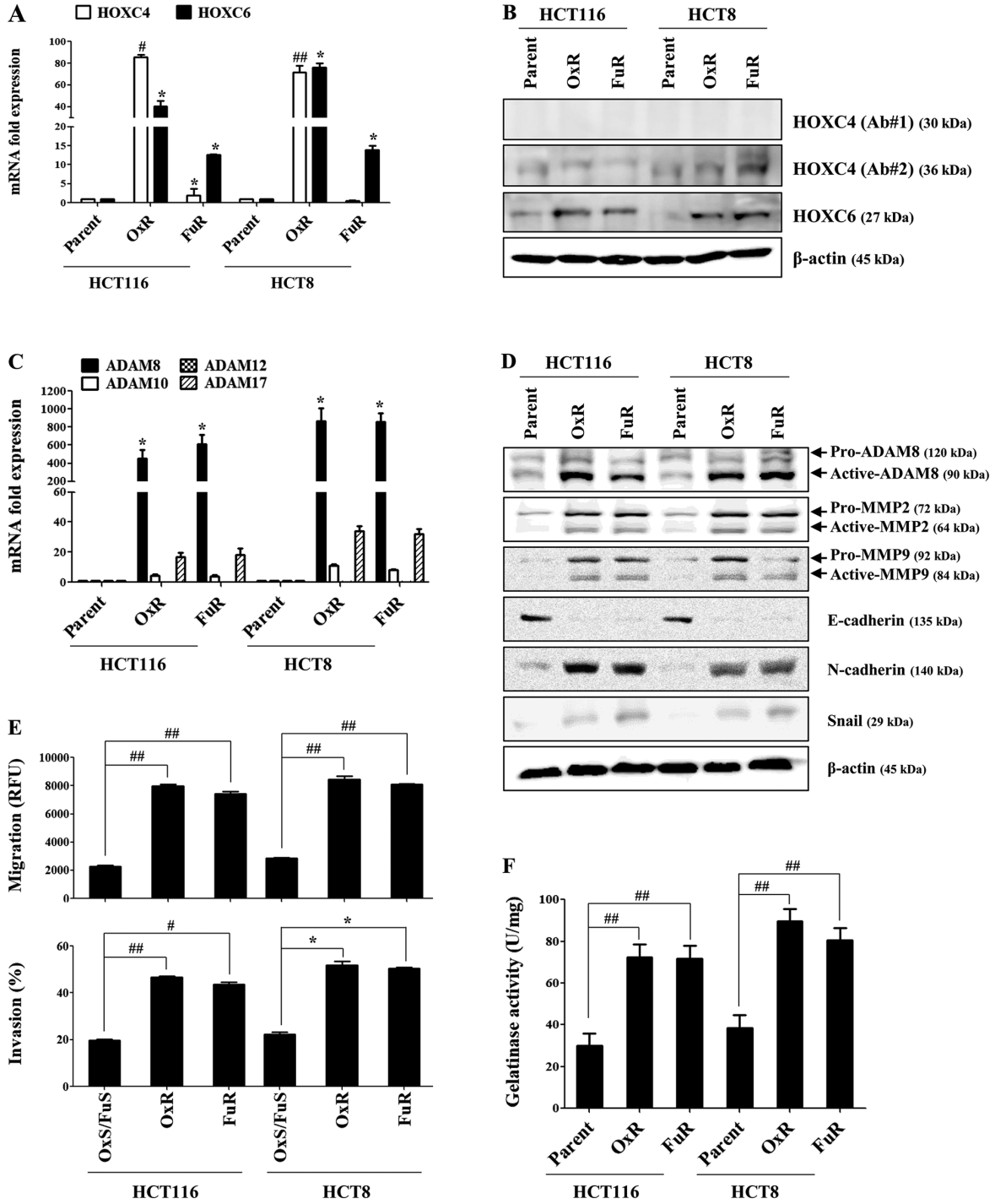

Figure 2. Expressions of HOXC6 and ADAM8 are significantly increased in chemoresistant colon cancer cells. OxR or FuR colon cancer and parent cells were seeded into 6-well plates ($1.5x10^5$/well) and cultured for 24 h. (A and C) Quantitative real-time PCR was performed to determine the relative expression of HOXC4, HOXC6, ADAM8, ADAM10, ADAM12 and ADAM17. (B and D) The total protein of each group was subjected to western blot analysis with the indicated antibodies. β-actin served as an internal control. (E) The migration and invasion of cells were detected using tumor transendothelial migration and BME cell invasion assay kits, respectively. (F) The gelatinase activity of cells was measured using a gelatinase assay kit. Data are presented as the mean ± SD of three independent experiments. *P<0.05, $^\#$P<0.005 and $^{\#\#}$P<0.001 vs. resistant cells. HOX, homeobox; ADAM, A disintegrin and metalloproteinase domain-containing 8; OxR, oxaliplatin resistant; FuR, 5-Fu-resistant; p, phosphorylated.

the suppression of ADAM10 and ADAM17 using specific inhibitors had no effect on the ADAM8-induced metastatic activity of chemoresistant colon cancer cells (Fig. 6C and D). These results suggest that TrkB/C activation and its downstream ERK-mediated HOXC6 signaling is one of the regulatory pathways that enhance the metastasis of drug-resistant colon cancer cells through the stimulation of ADAM8/MMP activity.

Discussion

Pharmacological inhibition of Trk significantly suppressed the migratory and invasive activity of drug-resistant colon cancer cells through the blocking the metalloproteinase activity. The inhibition of TrkB in lung squamous cell carcinoma not only prevents tumor cell invasion but also enhances

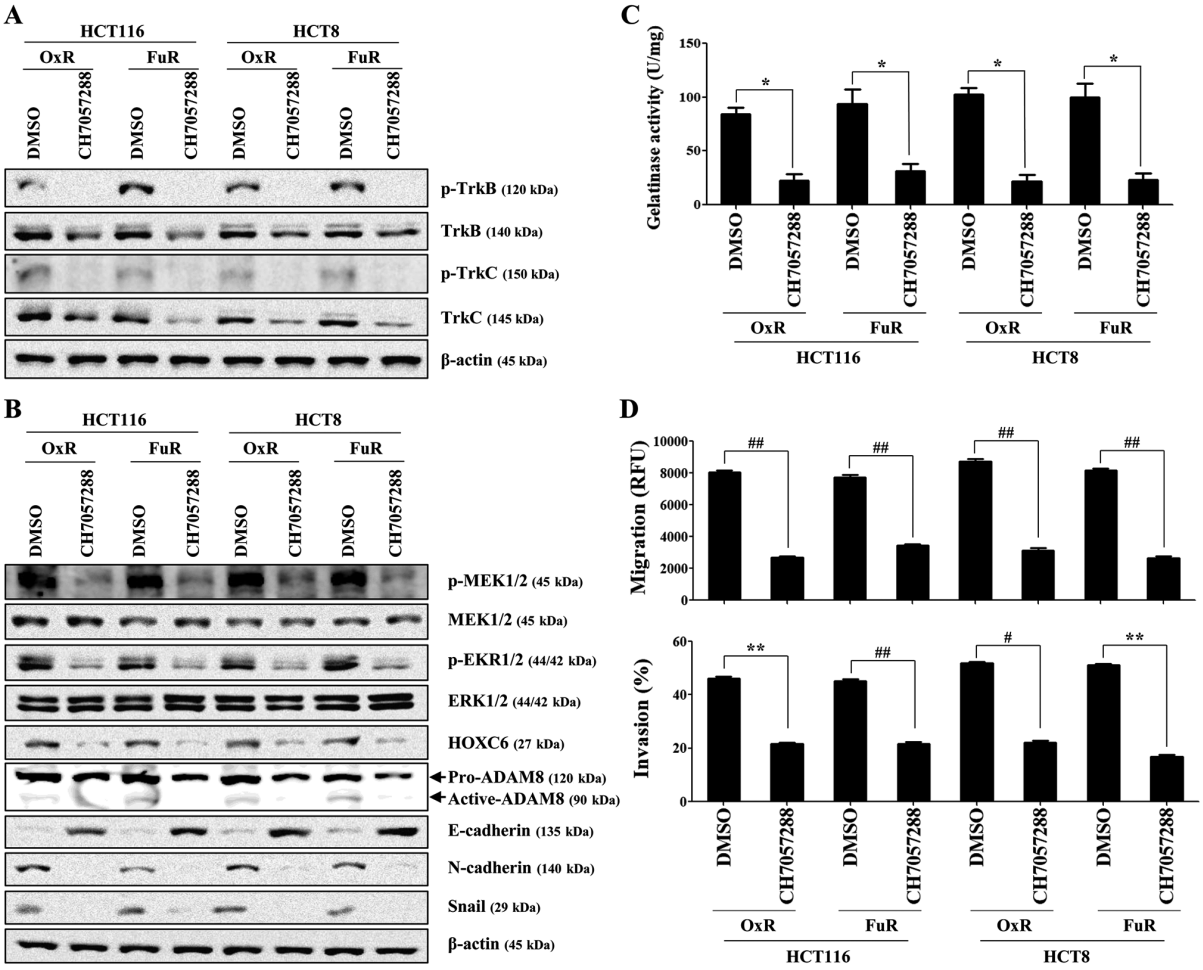

Figure 3. TrkB and TrkC activity mediates the upregulation of MEK and ERK during chemoresistant colon cancer cell metastasis. OxR or FuR colon cancer cells were seeded into 6-well plates ($1.5x10^5$/well) and treated with 2 μM CH7057288 for 24 h. (A and B) Total cell lysates were immunoblotted with the indicated antibodies. β-actin served as an internal control. (C) The gelatinase activity of cells was measured using a gelatinase assay kit. (D) The migration and invasion of cells was determined using tumor transendothelial migration and BME cell invasion assay kits, respectively. Each value represents the mean ± SD of three independent experiments. *P<0.05, **P<0.01, $^{\#}$P<0.005 and $^{\#\#}$P<0.001 as indicated. Trk, tropomyosin receptor kinase; OxR, oxaliplatin resistant; FuR, 5-Fu-resistant; HOX, homeobox; ADAM, A disintegrin and metalloproteinase domain-containing 8; p, phosphorylated.

the sensitivity to epidermal growth factor receptor (EGFR) inhibitors (30). The overexpression of TrkB/C and ADAM8 is also correlated with increased tumor invasion and poor prognosis of colon cancer patients (8,26). Therefore, it is important to understand the potential relationship between ADAM and TrkB/C in drug-resistant colon cancer cells. The overexpression of HOXC6 not only regulates chemoresistance through the upregulation of multidrug resistance protein 1 (MDR1) (18) but also triggers the metastasis of gastric cancer by activating MMP9 (31). Although higher HOXC6 expression is detected in metastatic colorectal cancer tissues than that of the primary cancer region (32), the exact function and relationship with other signaling pathways, particularly in drug-resistant colon cancer cells, are largely unknown. In this study, we have shown that TrkB/C-mediated MEK/ERK activation contributed to the stimulation of the HOXC6-mediated ADAM8 expression, resulting in the enhanced migratory and invasive activity of drug-resistant colon cancer cells. These results suggest that the TrkB/C-mediated HOXC6/ADAM8 signaling pathway is one of the regulatory factors that promote the metastasis of chemotherapy-resistant cancer cells (Fig. 6E).

Recent studies have shown that estrogen-induced BDNF in astrocytes activates the TrkB-AKT/ERK pathway, triggering the brain metastasis of triple-negative breast cancer (33). BDNF binding to its receptor, especially TrkB, plays a critical role in promoting proliferation, survival, and migration in various cancers, including colorectal cancer (34). BDNF-TrkB signaling pathway is well known for connection with nervous system development. However, the role or even expression of TrkB/C in chemoresistant colon cancer cells is still unknown. The mRNA expression of TrkB/C in drug-resistant HCT116 cells was significantly higher than that of chemoresistant HCT8 cells. Furthermore, we observed that the protein expression of Trk family has changed in cell-type dependent manner. These results suggest that several regulatory mechanisms at each step, such as mRNA and post-translational stage, work to control the expression of Trk family in colon cancer cells. It is necessary to investigate what controlling processes adjust the level of TrkB family in drug-resistant colon cancer cells in further study. Targeted inhibition of TrkB/C with CH7057288 suppressed the activation of MEK/ERK and downstream signaling pathways in this study. In addition, inhibiting the phosphorylation of ERK with PD98059 effec-

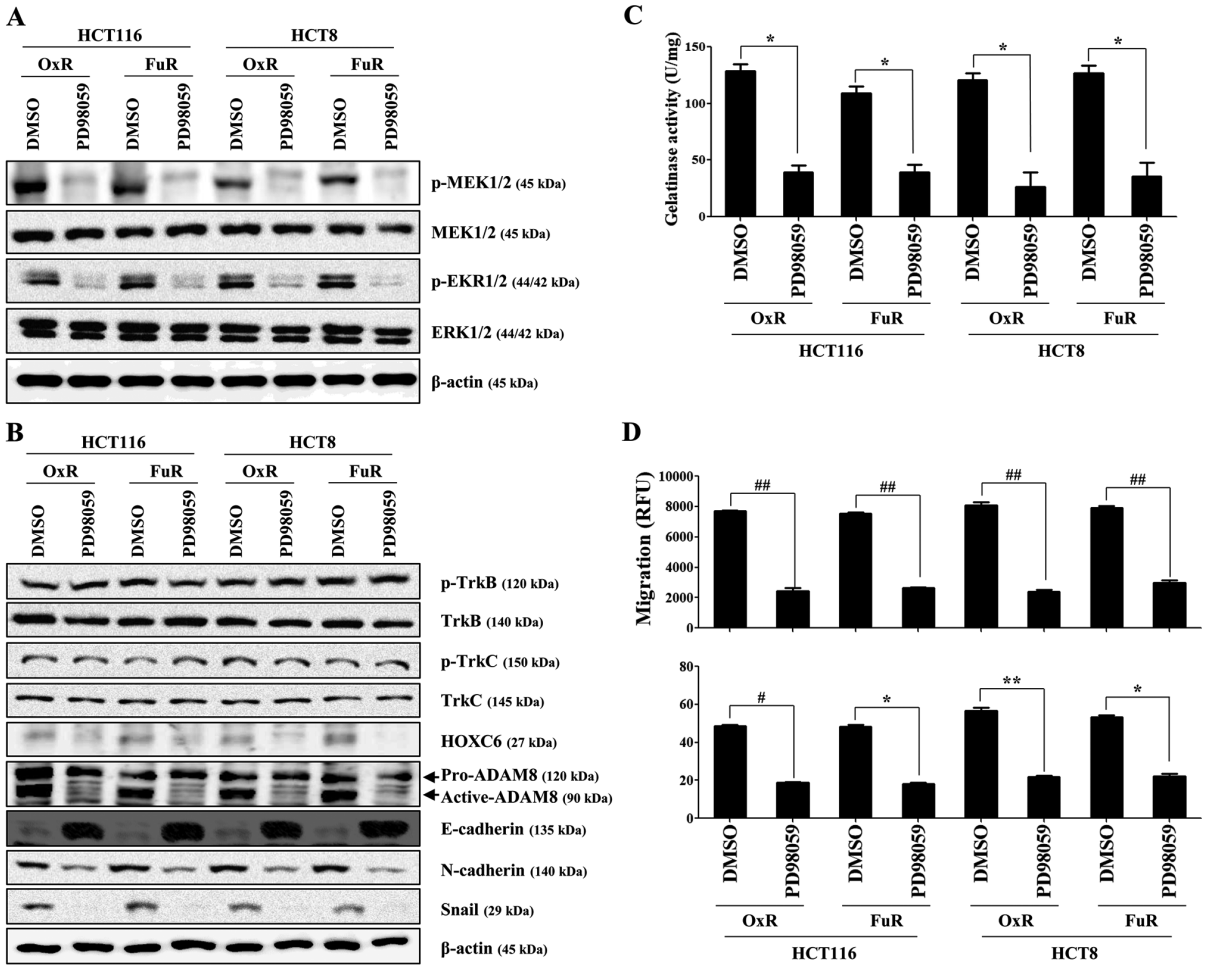

Figure 4. MEK/ERK activation induces HOXC6 and ADAM8 during chemoresistant colon cancer cell metastasis. OxR or FuR colon cancer cells were seeded into 6-well plates ($1.5x10^5$/well) and treated with 20 μM PD98059 for 24 h. (A and B) Total cell lysates were immunoblotted with the indicated antibodies. β-actin served as an internal control. (C) The gelatinase activity of cells was measured using the gelatinase assay kit. (D) The migration and invasion of cells was detected using tumor transendothelial migration and BME cell invasion assay kits, respectively. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01, $^{\#}$P<0.005 and $^{\#\#}$P<0.001 as indicated. HOX, homeobox; ADAM, A disintegrin and metalloproteinase domain-containing 8; OxR, oxaliplatin resistant; FuR, 5-Fu-resistant; Trk, tropomyosin receptor kinase; p, phosphorylated.

tively prevented the expression of HOXC6 and ADAM8 and the MMP-mediated migratory activity of the chemoresistant colon cancer cells. These results suggest that TrkB/C-mediated ERK activation plays an important role in the regulation of HOXC6 and ADAM8 activity.

The aberrant expression of HOX family genes is reported in various cancers, including colorectal cancer (17). HOXA9 expression is significantly upregulated in colon cancer tissues than in non-cancer areas and is closely related to increased lymph node metastasis (35). In addition, upregulation of HOXC6 triggers *MMP9* gene expression to promote the cell invasion of gastric cancer (31). However, the mechanism that induces HOXC6 expression and its downstream targets to enhance cancer metastasis remains unknown. Although HOXC6 expression induces MAPK activation to promote the metastasis of glioblastoma cells (16), we report for the first time that TrkB/C-mediated ERK activation may be one of the critical regulatory signaling pathways that trigger HOXC6 expression in this study. Furthermore, pharmacological inhibition of TrkB/C and ERK activation markedly reduced HOXC6 expression, but HOXC6 downregulation using siRNA did not affect the TrkB/C-mediated ERK signaling pathway in chemoresistant colon cancer cells. Despite upregulation of HOXC4 mRNA level, HOXC4 protein was barely detected in drug-resistant colon cancers. We can predict the various reasons why HOXC4 protein is downregulated in chemoresistant colon cancer cells, such as post-translational modification and miRNA-mediated regulation. The roles and controlling mechanisms of HOXC4 in drug-resistant colon cancer cells need to be investigated in future study. These results suggest that the TrkB/C-ERK pathway in drug-resistant colon cancer cells plays a critical role in the activation of HOXC6 and downstream signaling pathways.

Cancer cell metastasis requires several processes; these include proteolytic activity against adhesion molecules, penetration of the basement membrane by matrix remodeling, and angiogenesis (36,37). ADAM8-induced metalloprotease activation facilitates matrix remodeling, resulting in enhanced metastasis of breast cancer cells into the brain (25). A high level of ADAM8 expression in astrocytoma is closely related to increased tumor invasiveness through the activation of protease activity (23). The overexpression of ADAM10 and ADAM17 are connected to cancer cell proliferation, invasion, and the develop-

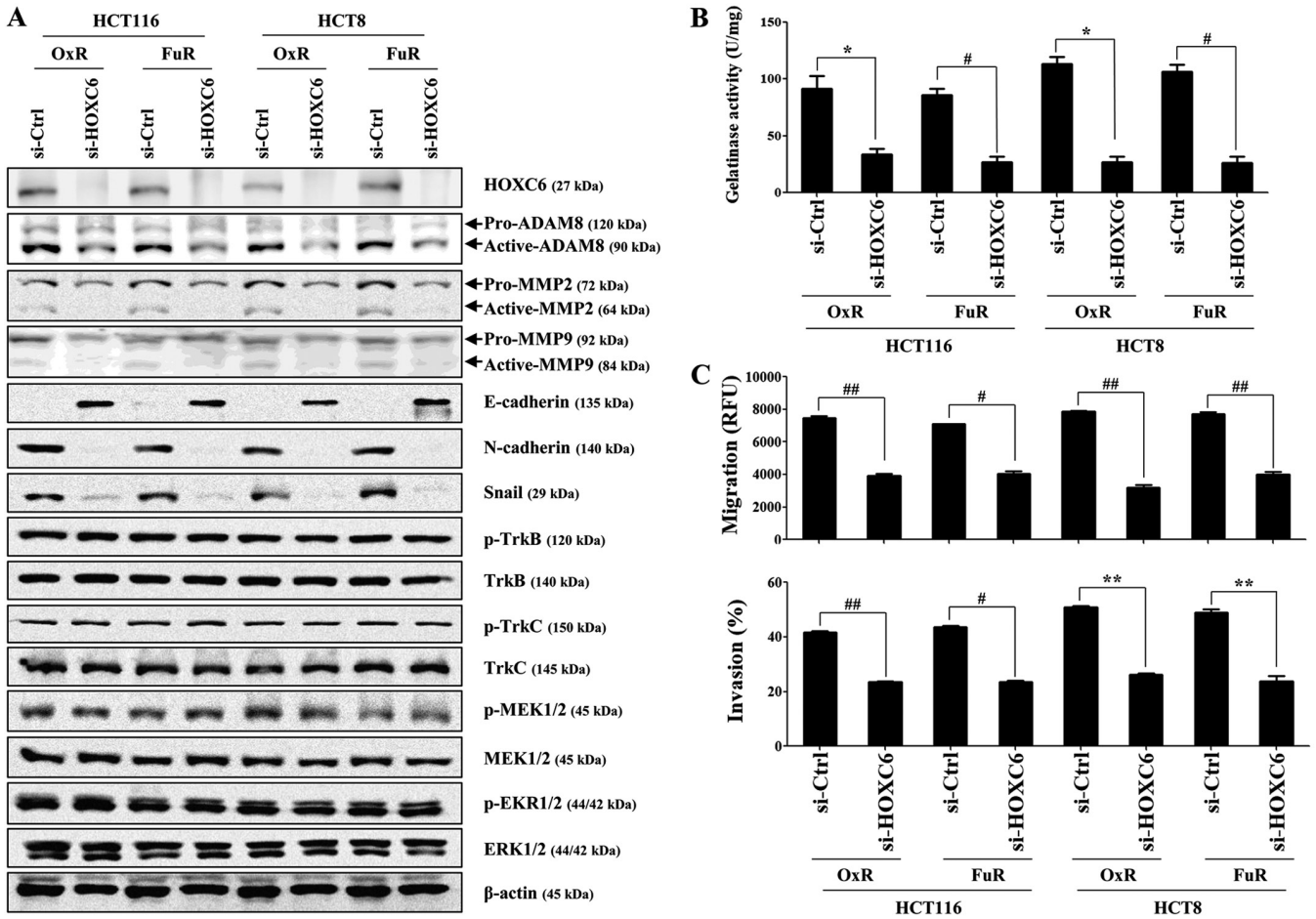

Figure 5. HOXC6 knockdown in OxR or FuR colon cancer cells decreases cell invasion and downregulates ADAM8 activation and gelatinase activity. OxR or FuR colon cancer cells were seeded into 6-well plates ($1.5x10^5$/well) and grown overnight. Cells were transfected with small interfering RNA against HOXC6 or control for 48 h. (A) Total cell lysates were immunoblotted with the indicated antibodies. β-actin served as an internal control. (B) The gelatinase activity of cells was measured by the gelatinase assay kit. (C) The migration and invasion of cells was detected using tumor transendothelial migration and the BME cell invasion assay kits, respectively. Data are presented as the mean ± SD of the three independent experiments. *P<0.05, **P<0.01, $^{\#}$P<0.005 and $^{\#\#}$P<0.001 as indicated. HOX, homeobox; OxR, oxaliplatin resistant; FuR, 5-Fu-resistant; ADAM, A disintegrin and metalloproteinase domain-containing 8; Trk, tropomyosin receptor kinase; si, small interfering RNA; ctrl, control; p, phosphorylated.

ment of drug resistance (38,39). We have also reported that the downregulation of ADAM10 and ADAM17 by pretreatment with 2-deoxy-D-glucose (2-DG) sensitizes chemoresistant colon cancer cells to 5-Fu (40). Although exposure lipopolysaccharide (LPS) to TLR4 in colon cancer cells enhances the ADAM10- and ADAM17-induced migratory activity (21), we have reported that the levels of TLR4 in drug-resistant colon cancer cells are significantly decreased in recent study (29). Based on these results, we expected that different member of ADAM family might involve in the metastatic activity of drug-resistant colon cancer cells. Targeted inhibition of ADAM8 in drug-resistant HCT8 and HCT116 cells effectively prevented their migratory activity by suppressing MMP. However, pharmacological inhibition of ADAM10 and ADAM17 failed to suppress the activation of the TrkB/C-ERK-HOXC6 signaling pathway. In addition, pretreatment with GI254023X (ADAM10 inhibitor, 10 μM) and Marimastat (ADAM17 inhibitor, 50 nM) had no effect on ADAM8 expression and the migratory or invasive activity of drug-resistant colon cancer cells. ADAM8 stimulates the ERK signaling pathway, which activates MMP to enhance the invasiveness of pancreatic ductal adenocarcinoma (28). However, we have shown that ADAM8 activation is dependent on TrkB/C-ERK-mediated HOXC6 activation during the metastasis of drug-resistant colon cancer cells in this study. Since ADAM8 has an influence on the expression of MMP9 in breast cancer (25) and HOXC6 expression triggers metastatic activity of gastric cancer cells through the activation of MMP9 (31), we investigated the relationship between ADAM8 with HOXC6 and regulatory role in migration of chemoresistant colon cancer cells. In this study, targeted downregulation of HOXC6 using siRNA prevented the ADAM8 expression as well as MMP-mediated invasive activity. Furthermore, gene silencing of ADAM8 using siRNA significantly blocked the MMP activity, resulting in inhibiting the migration. Based on these results, ADAM8 might be one of the potential downstream targets in HOXC6 signaling pathway to promote the MMP9 activity for inducing cancer cell metastasis.

Taken together, our results suggest that the activation of TrkB/C followed by the stimulation of the downstream MEK/ERK signaling pathway promotes HOXC6-mediated ADAM8 activation to induce metastasis in chemoresistant colon cancer cells. Therefore, screening the levels of TrkB/C, HOXC6,

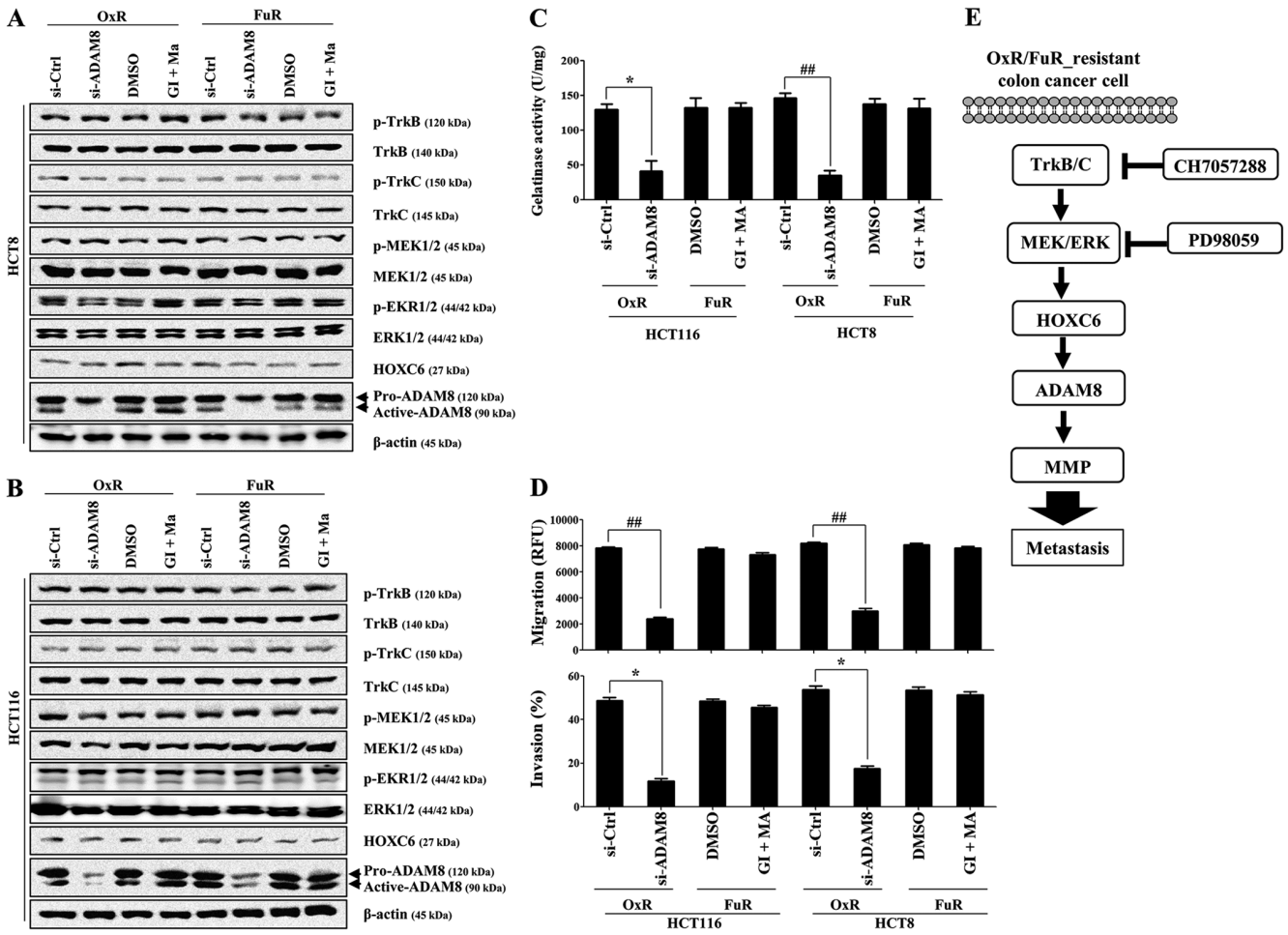

Figure 6. Activation of ADAM8 but not ADAM10 or ADAM17 regulates the invasion and gelatinase activity of metastatic drug-resistant colon cancer cells. OxR or FuR colon cancer cells were seeded into 6-well plates ($1.5x10^5$/well) and grown overnight. Cells were transfected with small interfering RNA against ADAM8 or control for 48 h, or treated with GI (10 μM) and MA (50 nM) for 24 h. (A and B) Total cell lysates were immunoblotted with the indicated antibodies. β-actin served as an internal control. (C) The gelatinase activity of cells was measured using the gelatinase assay kit. (D) The migration and invasion of cells were detected using tumor transendothelial migration and BME cell invasion assay kits, respectively. Data are presented as the mean ± SD of three independent experiments. *P<0.05 and $^{\#\#}$P<0.001 as indicated. (E) Schematic diagram of the intracellular signaling pathway in drug-resistant human colon cancer cells. ADAM8 expression contributed to the increased resistance and migratory activity of chemoresistant colon cancer cells through the activation of the TrkB/C-HOXC6 axis. ADAM, A disintegrin and metalloproteinase domain-containing 8; OxR, oxaliplatin resistant; FuR, 5-Fu-resistant; GI, ADAM10 inhibitor GI254023X; MA, ADAM17 inhibitor marimastat; Trk, tropomyosin receptor kinase; HOX, homeobox; si, small interfering RNA; Ctrl, control; p, phosphorylated.

and ADAM8 protein may be a new diagnostic measure to detect metastatic cancer cells in advanced colon cancer patients.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Authors' contributions

GBP and SC performed the experiments and analyzed the data. DK and YSY conceived and designed the present study, and wrote the manuscript. GBP and DK contributed to study design, coordinated the research and critically reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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