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ADAM9 is over-expressed in human ovarian clear cell carcinomas and suppresses cisplatin-induced cell death

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ADAMs (a disintegrin and metalloproteinases) are involved in various biological events such as cell adhesion, migration and invasion, membrane protein shedding and proteolysis. However, there have been no systematic studies on the expression of ADAMs in human ovarian carcinomas. We therefore examined mRNA expression of all the proteolytic ADAM species including ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, 30, 33 and ADAMDEC1 in human ovarian carcinomas, and found that prototype membrane-anchored ADAM9m, but not secreted isoform ADAM9s, is significantly over-expressed in carcinomas than in control non-neoplastic ovarian tissue. Among the histological subtypes of serous, endometrioid, mucinous and clear cell carcinomas, ADAM9m expression was highest in clear cell carcinomas. Immunohistochemistry showed that all the clear cell carcinoma samples displayed ADAM9m primarily on the carcinoma cell membrane. By immunoblotting, ADAM9m was detected mainly in an active form in the clear cell carcinoma tissues. When two clear cell carcinoma cell lines (RMG-I and TOV21G cells) with ADAM9m expression were treated with cisplatin, viability was significantly reduced and apoptosis increased in ADAM9m knockdown cells compared with mock transfectants. In addition, treatment of the cells with neutralizing anti-ADAM9m antibody significantly decreased viability compared with non-immune IgG, whereas ADAM9m over-expression significantly increased viability compared with mock transfectants. Our data show, to the best of our knowledge, for the first time, that ADAM9m is over-expressed in an activated form in human ovarian clear cell carcinomas, and suggest that ADAM9m plays a key role in cisplatin resistance.

KEYWORDS
a disintegrin and metalloproteinase 9, chemoresistance, cisplatin, clear cell carcinoma, ovarian carcinoma

1 | INTRODUCTION

Ovarian carcinomas, the most lethal gynecological malignancy, are histologically subdivided into serous, endometrioid, mucinous, clear cell, Brenner (urothelial) and undifferentiated carcinomas.[1] These subtypes show distinct differences not only in microscopic appearance but also in clinical behavior and molecular background.[1,2] Among them, clear cell carcinoma is characterized by resistance to

standard platinum- and taxane-based chemotherapy for ovarian carcinomas.[3,4] Although the incidence rate of clear cell carcinoma in Western countries is low ranging from 1% to 12%,[3,5] clear cell carcinoma is the second most common subtype (15%-25%),[3,6] following serous carcinoma (36%) in Japan.[6] Clear cell carcinoma patients at an advanced stage or with recurrent carcinoma usually take an unfavorable course mainly because of chemoresistance,[3,4,7] which is explained by mechanisms such as decreased drug accumulation, increased drug detoxification, and increased DNA repair activity.[7-10] However, the details underlying resistance to chemotherapy in clear cell carcinoma have not been clarified.

Members of the ADAM (a disintegrin and metalloproteinase) gene family are multifunctional proteins and involved in various pathophysiological events such as cell adhesion and migration, cell fusion, angiogenesis, and cancer cell proliferation and progression.[11-13] They are composed of 13 proteolytic ADAMs and eight non-proteolytic ADAMs.[11-13] Proteolytic ADAMs are transmembrane proteins comprising propeptide, metalloproteinase, disintegrin-like, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains, and share the metalloproteinase domain with that of matrix metalloproteinases.[11,13-15] Some of the members such as ADAM9, ADAM12 and ADAM28 have short-secreted splice variants besides prototype membrane-anchored forms: ADAM9s and ADAM9m (ADAM9-L),[16] ADAM12s and ADAM12m (ADAM12-L),[17] and ADAM28s and ADAM28m.[18] Previous studies on human ovarian carcinomas showed over-expression of ADAM10,[19] ADAM12[20] and ADAM17.[21] ADAM10 and ADAM17 are reported to have a key role in shedding of the adhesion molecule L1 (CD171) and heparin-binding EGF-like growth factor (HB-EGF), respectively.[19,21] ADAM12 is associated with clinical variables and poor prognosis of patients with high-grade serous ovarian carcinoma.[20] Expression of ADAM9 in ovarian carcinomas, albeit not significantly different from control, is also described without any comments on the function.[21] However, there have been no systematic studies on the expression profiles of ADAM family members in human ovarian carcinomas that are classified to histological subtypes. In addition, little or no information is available for the involvement of ADAM species in chemoresistance in ovarian carcinomas.

In the present study, we examined mRNA expression of all the proteolytic ADAM members in human ovarian carcinoma tissues, and found that only ADAM9m is significantly over-expressed in ovarian carcinomas, showing the highest expression in clear cell carcinoma. Our in vitro experiments also provided data that ADAM9m is involved in resistance to cisplatin in clear cell carcinoma cells.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and histology

Ovarian tumor samples (n = 48) from patients with primary ovarian carcinoma and control ovarian tissues (n = 13) stored in the Keio Women's Health Biobank (KWB) in Keio University School of Medicine were enrolled. All patients underwent surgery at Keio University Hospital and received no neoadjuvant chemotherapy prior to surgery. Written informed consent for experimental use of the samples was obtained from each patient registered in KWB. Ovarian carcinomas were pathologically classified according to the standard criteria of WHO histological classification of tumors of female reproductive organs.[1] Some parts of the carcinoma samples (n = 35) and non-neoplastic ovarian samples (n = 7) were snap-frozen, and stored at −80°C. Control non-neoplastic ovarian tissues were obtained from contralateral non-neoplastic ovaries in the ovarian carcinoma patients (n = 6) or from carcinoma-free ovaries in patients who underwent total hysterectomy and adnexectomy for International Federation of Gynecology and Obstetrics (FIGO) Stage I uterine corpus carcinoma (n = 7). The study protocol, which complied with the principles outlined in the Declaration of Helsinki, was approved by the Keio University School of Medicine Ethics Committee (Nos 20070081 and 20160326).

2.2 | RT-PCR and quantitative real-time PCR (qPCR)

cDNAs were prepared from isolated total RNA, and subjected to RT-PCR for examination of the 13 proteolytic ADAM species and housekeeping gene β-actin (Table S1) as described previously.[18] Relative expression levels of ADAM9m, ADAM10, ADAM15, ADAM17, ADAM28m and ADAM28s to β-actin were measured by qPCR with TaqMan probes (ABI Prism7000 Sequence Detection System; Applied Biosystems, Foster City, CA, USA) (Table S2). Although the primers for ADAM9 by qPCR detected both ADAM9m and ADAM9s, the data were considered to reflect ADAM9m expression as no or negligible expression of ADAM9s was found in all the ovarian carcinoma tissues examined by RT-PCR.

2.3 | Immunohistochemistry

Paraffin sections of the ovarian tissues were subjected to immunostaining with goat anti-human ADAM9 polyclonal antibody (AF939; R&D Systems, Minneapolis, MN, USA) or non-immune goat IgG (AB-108-C; R&D Systems) after blocking endogenous peroxidase and antigen retrieval by autoclaving, followed by reactions with biotinylated secondary antibody and 3-3′-diaminobenzidine.[18,22] Sections were counterstained with hematoxylin. ADAM9 immunostaining on cell membranes was scored from 0 to 3+ according to the 2007 ASCO/CAP HER-2/neu immunohistochemical scoring guidelines for breast cancer.[23] Briefly, no membranous immunostaining was scored 0; focal but not circumscribed membranous staining over 10% of tumor cells 1+; circumscribed membranous staining with moderate intensity over 10% of tumor cells 2+; and circumscribed membranous staining with strong intensity over 30% of tumor cells 3+. Intracytoplasmic immunostaining was also scored from 0 to 2+ as follows: score 0 for negative staining; score 1+ for weak staining; and score 2+ for strong staining. The sum of membranous and intracytoplasmic immunostaining scores was used as total

immunohistochemical score. MIB1-positive cell index was determined by immunostaining with anti-Ki-67 antibody (MIB1; DAKO A/S, Glostrup, Denmark).[18]

2.4 | Immunoblotting

Homogenate supernatants (50 μg/lane) of 19 ovarian carcinomas or ovarian carcinoma cell lines (20 μg/lane) were subjected to immunoblotting with anti-ADAM9 antibody to the C-terminal domain of human ADAM9m (#2099s; Cell Signaling Technology, Danvers, MA, USA) or anti-β-actin antibody (A5316; Sigma-Aldrich, St Louis, MO, USA), followed by reaction with ECL western blotting reagents (Thermo Fisher Scientific, Rockford, IL, USA). Densitometric analysis was done using ImageJ software (https://imagej.nih.gov/ij/).

2.5 | Cell cultures

RMG-I and TOV21G cell lines derived from human ovarian clear cell carcinomas were used after authentication by JCRB cell bank (Osaka, Japan; certification number, KBN0352). RMG-I cells were established by the Obstetric and Gynecologic Department, Keio University School of Medicine, and TOV21G cells were purchased from ATCC (Manassas, VA, USA). RMG-I and TOV21G cells were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) and DMEM/F-12 (Sigma-Aldrich) supplemented with 15% FBS and antibiotics, respectively.

2.6 | Knockdown of ADAM9 by shRNAs and overexpression of ADAM9m

The 5 different lentiviral vectors for ADAM9 shRNA and non-targeting shRNA vectors (mock) were purchased from Sigma-Aldrich. As two of them (sh1 and sh2) efficiently suppressed the expression, they were used for the experiments. The encoding sequences of the shRNAs were as follows: sh1, 5′-CCGGAGAGAAGTTCCTATATATCTCGAGATATATAGGAACTTCTCTGGGTTTTTG-3′; sh2, 5′-CCGGGCTAGTTCTAAAGAAAGGTTTCTCGAGAAACCTTTCTTTAGAACTAGCTTTTTG-3′. Stable transfectants were selected as described previously.[22] ADAM9m was over-expressed in TOV21G cells by transfection with pcDNA3.1 vectors encoding ADAM9m (Clone ID, OHu18124D; Genscript, Piscataway, NJ, USA) or mock pcDNA3.1 vectors, and the expression was examined by RT-PCR.

2.7 | Analyses of migration and activation of EGF receptor (EGFR)

RMG-I and TOV21G cells transfected with ADAM9m shRNA or mock vectors were grown to confluence and scratch-wounded with a 200-μL pipette tip. They were allowed to migrate in the medium containing 5 and 0.5 mmol/L hydroxyurea (Sigma-Aldrich), respectively. Activation of EGFR in the transfectants was examined by immunoblotting with anti-phosphoEGFR antibody (sc-12351R; Santa Cruz Biotechnology, Dallas, TX, USA) or anti-EGFR antibody (sc-03; Santa Cruz Biotechnology).

2.8 | MTT assay

RMG-I and TOV21G cells transfected with shRNA or mock vectors were treated with different concentrations of cisplatin (Sigma Aldrich) ranging from 0 to 50 μmol/L for up to 48 hours, and the viability was measured using MTT cell count kit (Nacalai Tesque, Kyoto, Japan). By preliminary experiments, LD50 and treatment times were determined to be 20 μmol/L for 48 hours for RMG-I cells and 15 μmol/L for 24 hours for TOV21G cells. They were also treated with neutralizing anti-human ADAM9 antibody specific to ectodomain (5 μg/mL, MAB939; R&D Systems)[24] or non-immune mouse IgG (5 μg/mL; BD Biosciences Pharmingen, Franklin Lakes, NJ, USA) for 1.5 hours prior to cisplatin treatment.

2.9 | Apoptosis assay

shRNA-transfected RMG-I and TOV21G cells were treated with LD50 concentrations of cisplatin. Both floating cells and detached cells by trypsin treatment were collected, stained with Annexin V and propidium iodide (PI) using an Annexin V FITC kit (Medical & Biological Laboratories, Nagano, Japan), and then subjected to flow cytometry (XL-MCL; Beckman Coulter, Miami, MA, USA). Results of flow cytometric analysis were analyzed on FlowJo software (ver. 7.6.5).

2.10 | Statistical analysis

Data between the two independent groups were determined by Mann-Whitney *U* test, and results of MTT and apoptosis assays were calculated by Student's *t* test. For comparison of more than 2 groups, values were corrected with Bonferroni's multiple comparison methods. Log-rank test and Kaplan-Meier method were used for survival analyses. *P*-values <.05 were considered to be significant.

3 | RESULTS

3.1 | mRNA expression of proteolytic ADAM species in human ovarian carcinomas

mRNA expression of ADAM8, ADAM9m, ADAM9s, ADAM10, ADAM12m, ADAM12s, ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28m, ADAM28s, ADAM30, ADAM33 and ADAMDEC1 was screened by RT-PCR in serous (n = 4), endometrioid (n = 3), mucinous (n = 3) and clear cell carcinomas (n = 4), and control non-neoplastic ovarian tissues (n = 3). There was no or negligible expression of ADAM9s, ADAM12s, ADAM33 and ADAMDEC1 in the carcinoma or the non-neoplastic tissues, and expression of ADAM8, ADAM12m, ADAM19, ADAM20, ADAM21 and ADAM30 was observed in less than ~50% of the carcinoma samples (Figure 1).

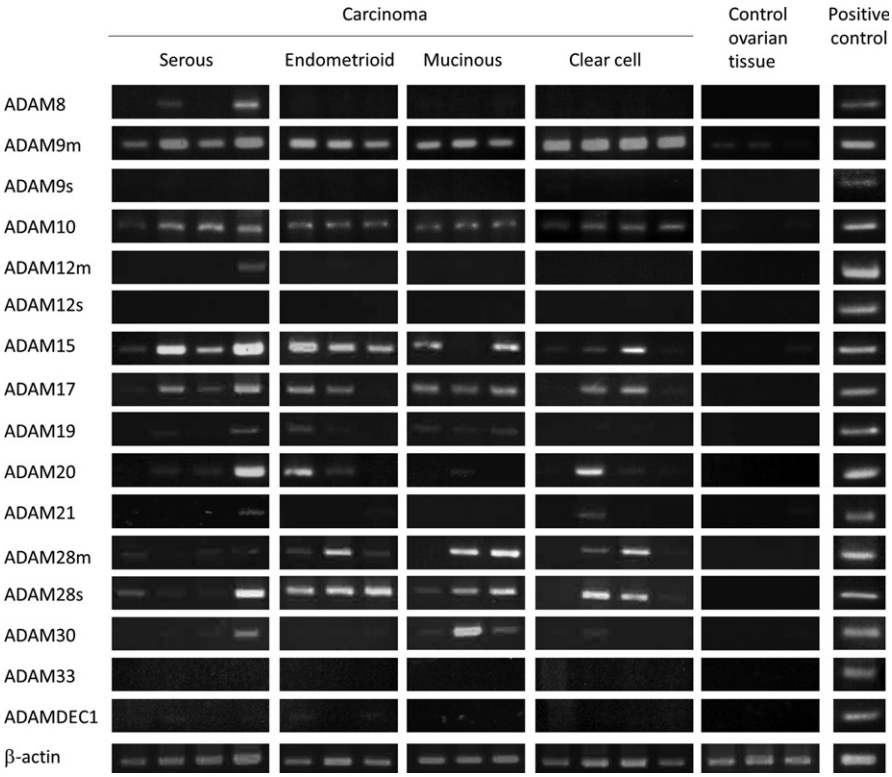

FIGURE 1 RT-PCR analysis of all the proteolytic ADAM (a disintegrin and metalloproteinases) species in the four ovarian carcinoma subtypes and control non-neoplastic ovarian tissues. Positive control for each ADAM species shows RT-PCR using mRNAs isolated from various human carcinoma cell lines

In contrast, ADAM9m, ADAM10, ADAM15, ADAM17, ADAM28m and ADAM28s were expressed in more than 70% of the carcinoma tissues, and the expression of these ADAM species appeared to be high in the carcinomas and only weak in the non-neoplastic ovarian tissues (Figure 1). Thus, we further analyzed the expression levels of these ADAM species in a larger number of ovarian carcinoma and control ovarian tissues by qPCR.

3.2 | Over-expression of ADAM9m and its correlations with clinicopathological factors

Expression levels of ADAM9m, ADAM10, ADAM15, ADAM17, ADAM28m and ADAM28s were compared by setting the average level in the control samples as 1.0. Among the ADAM species examined, only the ADAM9m level was significantly 3.1-fold higher in the carcinoma tissues (3.11 ± 2.52; mean ± SD; n = 35) than in the control non-neoplastic ovarian tissues (1.00 ± 0.40; n = 7) ($P < .01$) (Figure 2A). Expression level of ADAM28m appeared to be higher in the carcinoma samples (4.14 ± 4.94; n = 30) than in the control samples (1.00 ± 0.64; n = 7), although no significant difference was obtained between the two groups ($P = .068$) (Figure 2A). Expression levels of ADAM10, ADAM15, ADAM17 and ADAM28s were almost similar between the carcinoma and the control non-neoplastic samples (Figure 2A). Therefore, we further analyzed ADAM9m expression levels by focusing on the four histological subtypes of ovarian carcinomas. As shown in Figure 2B and Table S3, the level in the clear cell carcinomas (4.52 ± 2.79; n = 13), all the samples of which expressed ADAM9m, was the highest, and significantly higher than that in the control group (1.00 ± 0.40; n = 7). The levels were also significantly higher in the endometrioid (2.22 ± 0.93; n = 6) and mucinous carcinomas (3.68 ± 3.51; n = 5), but not in the serous carcinomas (1.67 ± 1.19; n = 11), than in the control group (Figure 2B; Table S2). Expression of ADAM9m was significantly ~2-fold higher in the clear cell carcinomas (4.52 ± 2.79; n = 13) than in the non-clear cell carcinomas (2.27 ± 1.97; n = 22) ($P < .01$) (Figure 2C). ADAM9m expression level was also significantly higher in Grade 3 ovarian carcinomas (3.91 ± 2.69; n = 17) than in Grade 1/2 carcinomas (2.36 ± 2.16; n = 18) ($P < .05$) (Table S3). However, no positive correlations were observed between the expression levels of ADAM9m, ADAM10, ADAM15, ADAM17, ADAM28m and ADAM28s and clinicopathological parameters including age at operation, vascular and/or lymphatic invasion, lymph node metastasis, MIB1-positive cell index and clinical stages of the disease (Table S3 and data not shown for ADAM10, ADAM15, ADAM17, ADAM28m or ADAM28s). In contrast, overall survival tended to be shorter in the ADAM9m-high patient group than in the ADAM9m-low group (log-rank test, $P = .0594$; Figure S1).

3.3 | Protein expression of ADAM9m in ovarian carcinoma and non-neoplastic ovarian tissues

Immunohistochemistry with anti-ADAM9m antibody showed that ADAM9m was positively stained in the cytoplasm and/or on the cell membrane in 94% of the ovarian carcinoma samples (45/48 cases), whereas the control non-neoplastic ovarian tissues (6 cases) showed negligible immunostaining in the ovarian surface epithelial cells (Figure 3). No obvious immunostaining of

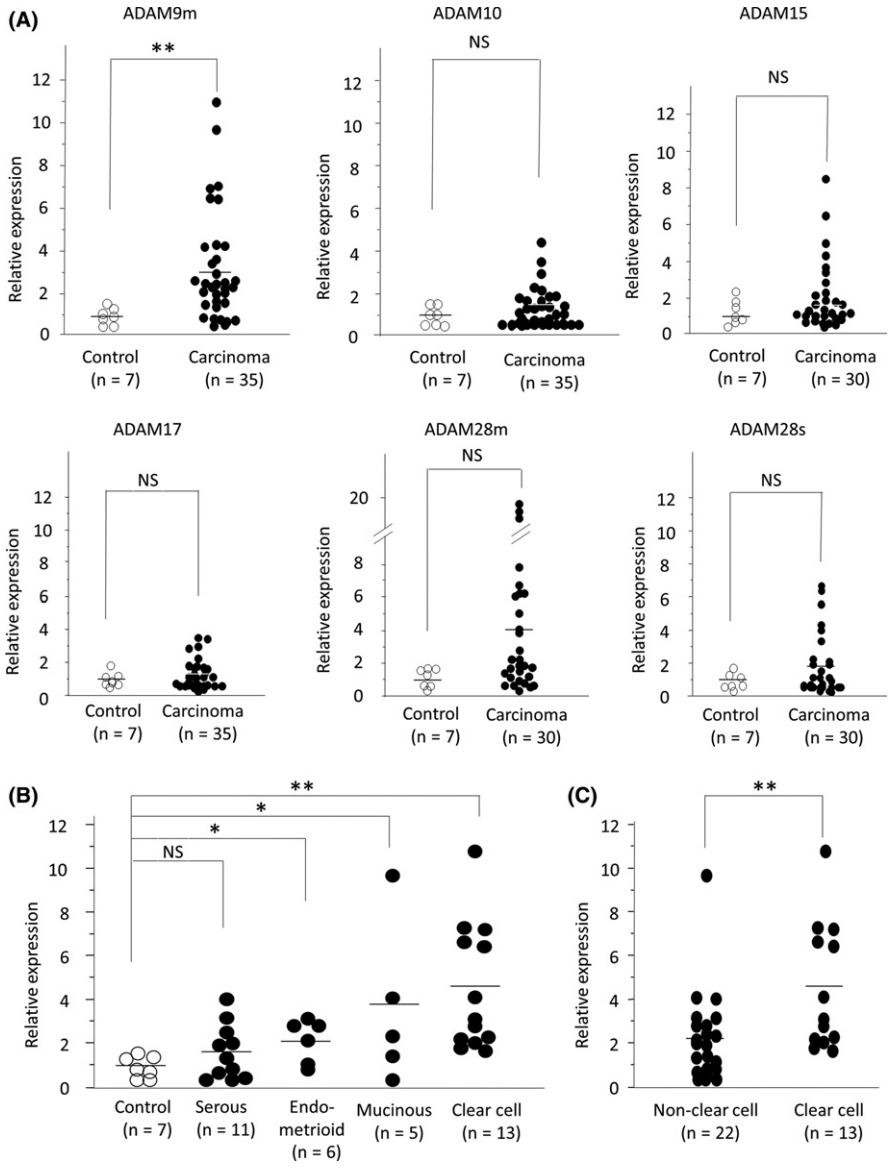

FIGURE 2 Quantitative real-time PCR (qPCR) analysis of the relative mRNA expression levels of ADAM (a disintegrin and metalloproteinase) species screened by RT-PCR and the expression levels of ADAM9m in four ovarian carcinoma subtypes. A, Expression levels of ADAM9m, ADAM10, ADAM15, ADAM17, ADA28m and ADAM28s in ovarian carcinoma and control non-neoplastic ovarian tissues. B, Expression levels of ADAM9m in ovarian carcinoma subtypes and control non-neoplastic ovarian tissue. C, Expression levels of ADAM9m between non-clear cell and clear cell carcinomas. Bars indicate mean values. *$P < .05$; **$P < .01$; NS, not significant

ADAM9m was observed in stromal cells of the carcinoma or control ovarian tissues, and non-immune IgG showed only background staining (Figure 3). Intracytoplasmic staining was observed in 71.4% of the serous carcinomas (10/14 cases), in 94.4% of the clear cell carcinoma (17/18 cases) and in 100% of the endometrioid carcinomas (10/10 cases) and the mucinous carcinomas (6/6 cases). Although the non-clear cell carcinomas had a weak and diffuse intracytoplasmic staining pattern (intensity score 1+), most of the clear cell carcinomas showed intracytoplasmic staining with a strong and coarse granular pattern (intensity score 2+) (Figure 3). Importantly, all the clear cell carcinomas (18/18 cases) showed membranous staining, which was characterized by focal membranous staining (membrane score 1+) (50%; 9/18 cases), circumscribed membranous staining with moderate intensity (membrane score 2+) (39%; 7/18 cases) or circumscribed membranous staining with strong intensity (membrane score 3+) (11%; 2/18 cases) (Figure 3). However, all cases of serous, endometrioid and mucinous carcinomas, with an exception of one mucinous carcinoma case, showed only focal circumscribed membranous staining (membrane score 1+) at the apical sites of glandular structures or no membranous staining (membrane score 0) (Figure 3).

When intensity score, membrane score and total score of ADAM9m were compared among the histological subtypes, all the scores appeared to be higher in the clear cell carcinomas (Figure 4A, C,E). Intensity, membrane and total scores were significantly higher in the clear cell carcinomas than in the non-clear cell carcinomas ($P < .05$ or $P < .001$) (Figure 4B,D,F). Total score of the ADAM9m immunostaining was also significantly higher in the G3 ovarian carcinomas compared with the G1/2 carcinomas ($P < .01$) (Table S4). However, there were no significant differences of the immunostaining scores in other clinicopathological parameters including age at operation, vascular and lymphatic invasion, nodal involvement, MIB1-positive cell index and FIGO stages of the disease between

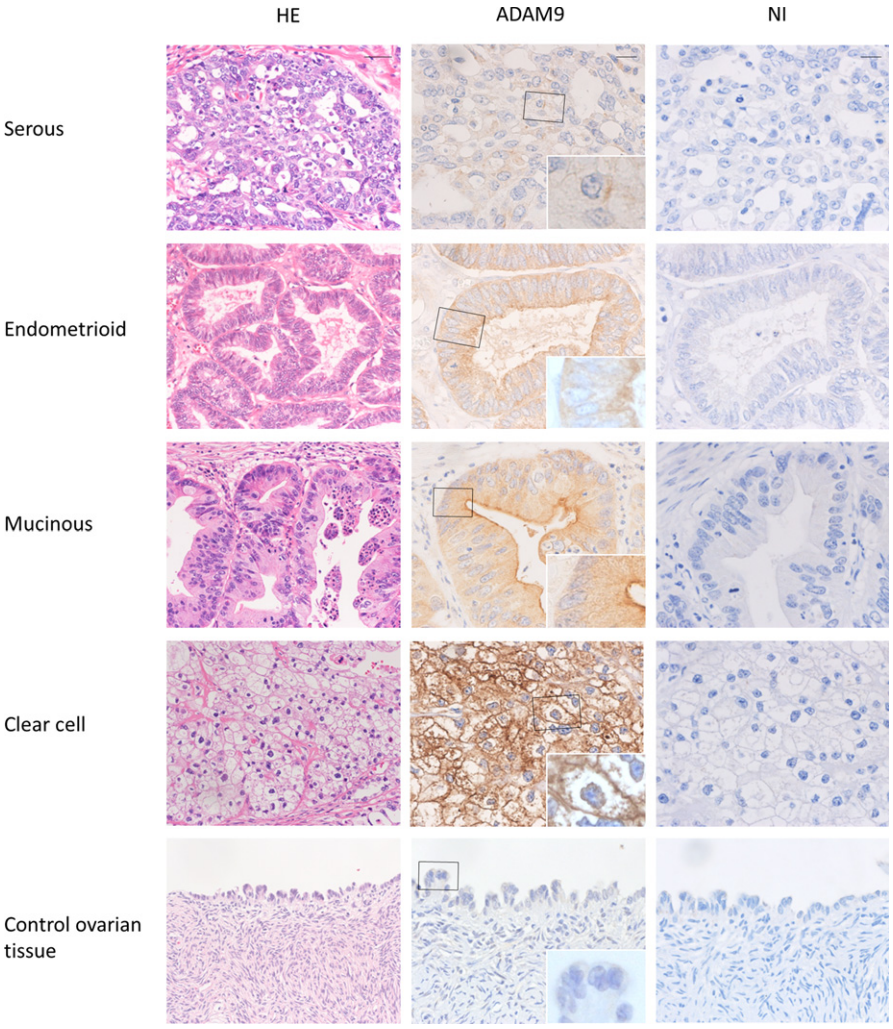

FIGURE 3 Immunohistochemical localization of ADAM9 in ovarian serous, endometrioid, mucinous, and clear cell carcinomas and control non-neoplastic ovarian tissue. Insets show high-power view of the rectangle areas of ADAM9 immunostaining. NI, negative control with non-immune IgG. Bar, 50 μm. Note the intense and circumscribed membranous staining with granular intracytoplasmic staining of ADAM9 in the clear cell carcinoma. ADAM, a disintegrin and metalloproteinase

the low-ADAM9 and the high-ADAM9 groups of clear cell carcinoma (data not shown).

ADAM9m protein expression in ovarian carcinoma tissues was confirmed by immunoblotting. Active ADAM9m of 74 kDa and/or precursor form of ADAM9m of 100 kDa were detected in 15 of 19 carcinoma tissues, whereas no definite immunoreactive bands were obtained with non-immune rabbit IgG (Figure S2A). Densitometric analysis of the immunoreactive bands showed that relative expression levels of ADAM9m were significantly higher in the clear cell carcinomas compared with the non-clear cell carcinomas ($P < .05$) (Figure S2B).

3.4 | Knockdown, blocking or over-expression of ADAM9m and their influences on cisplatin resistance in ovarian clear cell carcinoma cell lines

As clear cell carcinoma is commonly resistant to platinum-based chemotherapy,[3,7] we studied the possible involvement of ADAM9m on cisplatin resistance using human ovarian clear cell carcinoma cell lines, RMG-I and TOV21G cells. They expressed ADAM9m, ADAM10 and ADAM17, but not ADAM9s (Figure 5A). When the expression of ADAM9m was knocked down by shRNAs for ADAM9 (sh1 and sh2), ADAM9m protein expression was effectively reduced in RMG-I^{sh1} (44.7% ± 12.2%), RMG-I^{sh2} (49.4% ± 9.0%), TOV21G^{sh1} (10.7% ± 8.8%) and TOV21G^{sh2} (47.6% ± 22.8%) cells as compared to their mock transfectants (Figure 5B), although no changes in the expression of ADAM10 or ADAM17 were seen (Figure 5A). We first examined cell migration and EGFR phosphorylation, and found that cell migration activity was significantly increased in shRNA transfectants of both RMG-I and TOV21G cells compared with Mock transfectants (Figure 5C). In contrast, EGFR phosphorylation was significantly decreased in RMG-I^{sh1} and RMG-I^{sh2} cells compared with Mock transfectants (Figure 5D). A similar finding was seen in TOV21G^{sh1} and TOV21G^{sh2} cells, although no significant difference was obtained with the TOV21G^{sh2} cells (Figure 5D).

When these cells were treated with cisplatin at LD50 concentrations for each cell line, cell viability by MTT assay was significantly decreased in RMG-I^{sh1} (21.2% ± 9.4%) and RMG-I^{sh2} (31.4% ± 6.3%) compared with RMG-I^{Mock} (51.5% ± 6.4%) ($P < .001$) (Figure 6A). Similar results were obtained with TOV21G^{sh1} (21.4% ± 5.6%) and TOV21G^{sh2} cells (25.7% ± 9.4%) compared

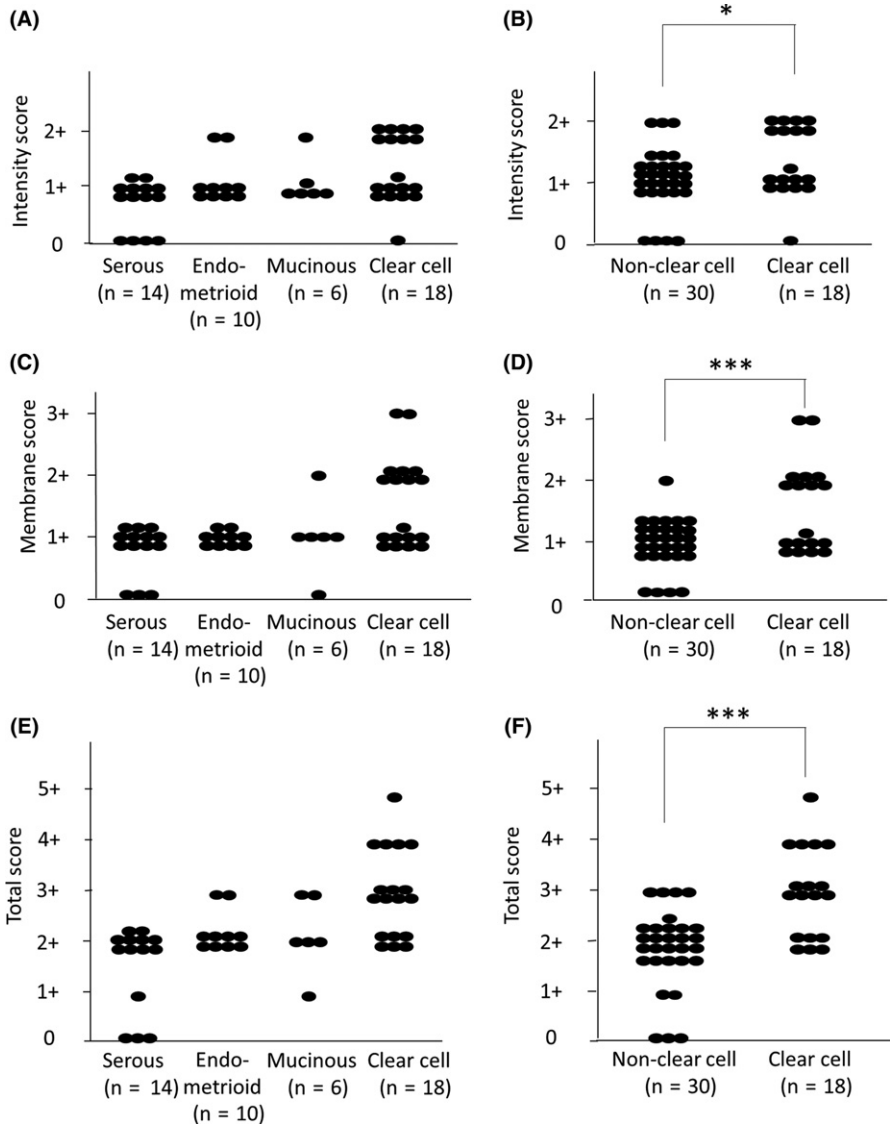

FIGURE 4 Intensity, membrane, and total scores of ADAM9 immunostaining in ovarian serous, endometrioid, mucinous, and clear cell carcinomas. A,C,E, Distribution of the intensity (0, 1+ and 2+), membrane (0 to 3+) and total scores (0 to 5+) of ADAM9 immunostaining. B,D,F, Distribution of the intensity, membrane and total scores of ADAM9 immunostaining in non-clear cell and clear cell carcinoma groups. *$P < .05$; ***$P < .001$. ADAM, a disintegrin and metalloproteinase

with TOC21G^{Mock} cells (50.3% ± 7.5%) ($P < .001$) (Figure 6A). Flow cytometric analysis indicated that numbers of Annexin V-positive and PI-positive cells (apoptotic cells) after cisplatin treatment were significantly increased in RMG-I^{sh1} (43.8% ± 6.2%) and RMG-I^{sh2} (40.9% ± 2.7%) cells compared to RMG-I^{Mock} cells (25.0% ± 2.6%) ($P < .001$) (Figure 6B). TOV21G cells also showed an increase in numbers of apoptotic cells in TOV21G^{sh1} (58.7% ± 16.1%) and TOV21G^{sh2} cells (48.5% ± 12.1%) compared to TOV21G^{Mock} cells (19.7% ± 5.5%) ($P < .01$) (Figure 6B). We then examined the effect of anti-ADAM9 antibody specific to the ectodomain of ADAM9m or ADAM9m over-expression on cisplatin cytotoxicity. The anti-ADAM9 antibody treatment significantly reduced the viability of RMG-I cells (24.6% ± 7.7%) compared with non-immune IgG-treated cells (48.0% ± 4.2%) and in TOV21G cells (22.1% ± 2.8%) compared with non-immune IgG-treated cells (46.0% ± 5.7%) ($P < .001$) (Figure 6C). However, the viability of cisplatin-treated TOV21G cells transfected with ADAM9m expression vectors (70.2% ± 13.6%) was significantly higher than Mock transfectants (43.0% ± 8.4%) ($P < .01$) (Figure 6D).

4 | DISCUSSION

In the present study, we have shown, to the best of our knowledge, for the first time, that active ADAM9m is over-expressed on carcinoma cell membranes in human ovarian clear cell carcinomas. Previous studies have shown that ovarian carcinomas over-express ADAM10,[19] ADAM12,[20] and ADAM17.[21] ADAM12 is reportedly highly expressed by high-grade serous carcinomas,[20] but no data on the histological subtype-specific expression of ADAM10 and ADAM17 are available.[19,21] Although Tanaka et al[21] described the expression of ADAM9, albeit not significantly different from the control, in ovarian cancers, the study provided no information about the expression profiles of ADAM9 in the 4 histological subtypes. In contrast, in the present study, we found that among the proteolytic ADAM species, only ADAM9m expression was significantly higher in the carcinomas, especially in the clear cell carcinomas than in the control samples. Reasons for the different expression profiles of these ADAM species are not clear at the present time. However, our data on the higher-level expression of ADAM9m in the carcinomas

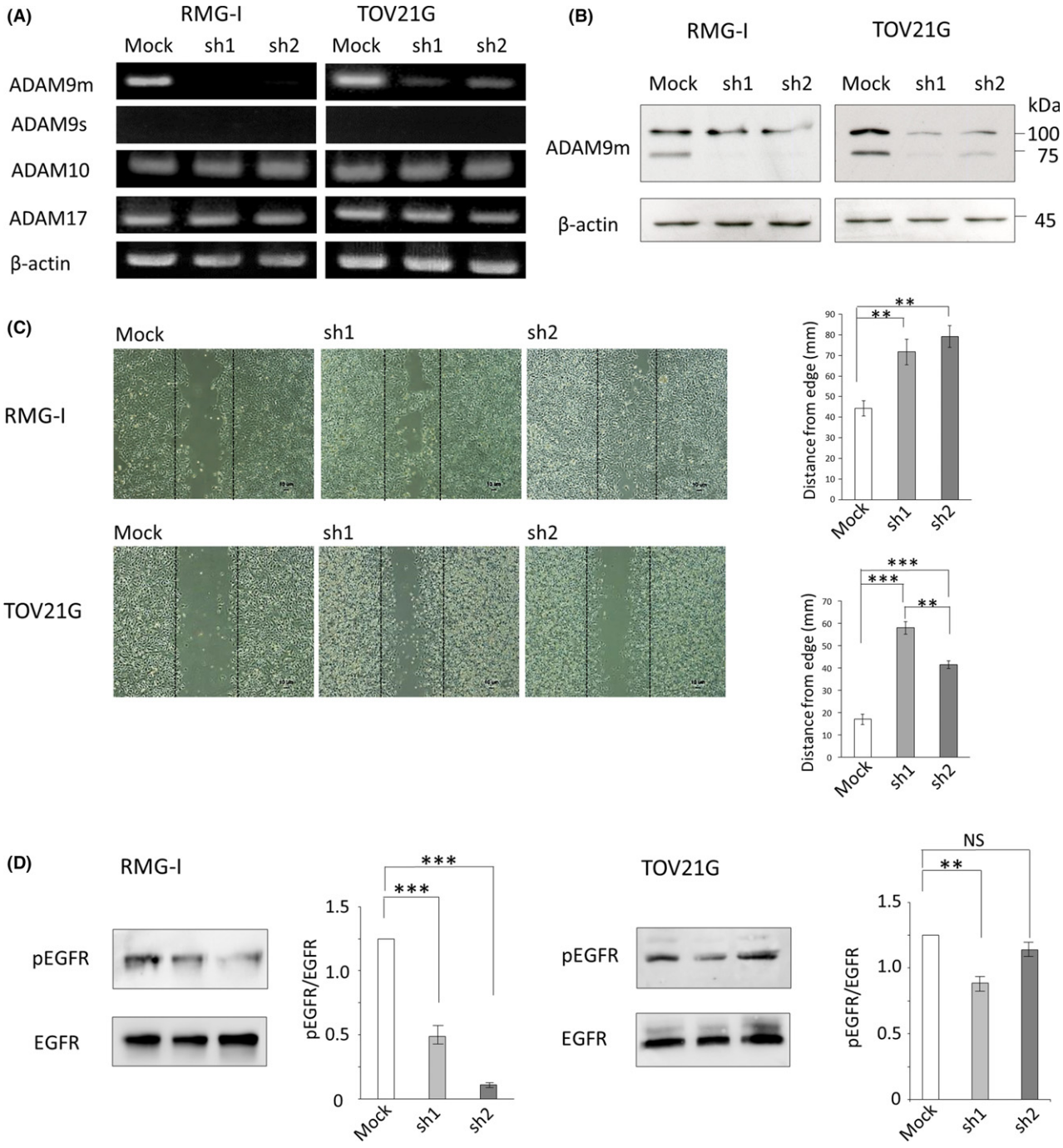

FIGURE 5 Knock-down of ADAM9m in human ovarian clear cell carcinoma cell lines (RMG-I and TOV21G) and its effect on cell migration and EGFR activation. A, mRNA expression of ADAM9m, ADAM9s, ADAM10 and ADAM17 in cells transfected with shRNAs (sh1 and sh2) for ADAM9 or non-targeting shRNA (Mock) vectors by RT-PCR. β-actin, a loading control. B, Protein expression of ADAM9m in cells transfected with sh1, sh2 or Mock vectors by immunoblotting. C, Migration assay of cells transfected with sh1, sh2 or Mock vectors. Migration activity was measured at 24 h after scratch-wounding. D, Activation of EGFR of cells transfected with sh1, sh2 or Mock vectors. Expression of phosphorylated EGFR (pEGFR) and EGFR was examined by immunoblotting. All assays were carried out in triplicate, and repeated 3 times. Bars, mean value ± SD. $^{**}P < .01$; $^{***}P < .001$; NS, not significant. ADAM, a disintegrin and metalloproteinase

may be ascribed, at least in part, to the high percentage of clear cell carcinoma cases in our samples (13 cases of total 35 ovarian carcinoma cases).

Epithelial ovarian carcinomas can be classified into major types designated as type I and type II carcinomas according to the distinctive clinicopathological and molecular genetic features.[1,2,25] Type I carcinomas are composed of low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas and are relatively stable genetically, whereas type II carcinomas comprise high-grade serous, high-grade endometrioid, malignant mixed mesodermal

tumors (carcinosarcomas) and undifferentiated carcinomas and all of them are aggressive, having a very high frequency of TP53 mutations.[1,2,25] Interestingly, high-level expression of ADAM9m was closely related to type I carcinomas (ie, clear cell, mucinous, and endometrioid carcinomas) in the present study. No or little information is available for the molecular mechanism of ADAM9m gene expression in ovarian carcinomas. However, our previous study on oncogene-transformed MDCK cells has shown that gene expression of ADAM9, ADAM10, ADAM12 and ADAM28 is induced by oncogene-modulated transformation.[26] As type I ovarian carcinomas are characterized by specific mutations such as ARID1A, PIK3CA, KRAS, BRAF and PTEN, which target several cell signaling pathways, and develop in a stepwise way from precursor lesions,[25] it is possible to speculate that the cell signaling pathways caused by these mutations may trigger ADAM9m gene expression after transformation to type I ovarian carcinomas. In contrast, in prostate carcinoma cell lines, exposure of the cells to stress conditions such as cell crowding, hypoxia, and hydrogen peroxide is known to promote ADAM9 expression, and reactive oxygen species (ROS) and/or hydrogen peroxide generated by the cell stress are thought to be responsible for the expression.[27] Reactive oxygen species (ROS) reportedly serves as a common downstream mediator for androgen- or serum starvation-induced ADAM9 expression in androgen receptor-positive prostate carcinoma cells.[28] Hydrogen peroxide is also known to promote the expression of ADAM9 in A549 lung adenocarcinoma cells.[24] Ovarian clear cell and endometrioid carcinomas are frequently associated with endometriosis[10,29,30] and the majority of them are considered to arise from pre-existing endometriosis under the tissue microenvironment with severe iron-induced oxidative stress.[31,32] Therefore, iron-induced ROS may be implicated in the stimulation of ADAM9m expression in ovarian clear cell and endometrioid carcinomas. However, further studies are definitely needed to demonstrate the hypothesis on ADAM9m over-expression in ovarian carcinomas.

Previous studies have shown over-expression of ADAM9 in various human carcinomas including breast,[33] lung,[34] prostate,[35] stomach,[36] pancreas,[37] liver,[38] and kidney cancers,[39] although most of these studies did not analyze the expression by focusing on its isoforms (ie, ADAM9m and ADAM9s). A recent study on human breast carcinoma tissues and cell lines disclosed that ADAM9m and ADAM9s play distinct roles in carcinoma cell migration by inhibiting and promoting the migration, respectively.[16] In the present study, we confirmed the inhibitory effect of ADAM9m on cell migration. ADAM9s is known to enhance adhesion of A549 lung adenocarcinoma cells to cultured endothelial cells and increase Matrigel invasion through binding to β1 integrin, showing the stimulative effect on cancer cell dissemination.[24] In the metastasis of colon carcinoma cells, ADAM9s expressed by hepatic stellate cells is reported to bind to α6β4 and/or α2β1 integrins of colon carcinoma cells and enhance carcinoma cell invasion through digestion of laminin in the liver.[40] One of the characteristics of ovarian clear cell carcinomas is relatively slow growth[3,4] and disease is presented at early stages.[3,4,10,41] Taken together, over-expression of ADAM9m, but not ADAM9s, seems to be in accordance with the less aggressive character of ovarian clear cell carcinomas at least in the early stages. Our results that ADAM9m expression showed no correlations with clinicopathological factors including MIB-1-positive cell index, lymphatic and vascular invasion, metastasis and clinical stages suggest that ADAM9m expressed by ovarian clear cell carcinoma cells plays a role different from cancer cell proliferation, invasion or metastasis.

Ovarian clear cell carcinomas are known to be mostly resistant to chemotherapy, and this chemoresistance is a major reason for the poor prognosis of patients with ovarian clear cell carcinoma.[4,7,10,41] Numerous cellular mechanisms contributing to resistance to chemotherapeutic drugs such as cisplatin have been proposed, including changes in cellular uptake and/or efflux of drugs, increased detoxification, inhibition of apoptosis and increased DNA repair.[4,7,10] As reducing cisplatin accumulation by cancer cells seems to be a major form of the resistance[42] and multi-drug resistance genes such as MDR-1, MRP-1, MRP-2 and ABCF2 are involved in efflux of cisplatin from the cells in many carcinoma cells including ovarian clear cell carcinoma cells,[7,43] we first examined the effect of ADAM9m on the expression of these multi-drug resistance genes by knocking down ADAM9m expression by shRNAs in ovarian clear cell carcinoma cell lines, and found no changes in their expression (Ueno M., et al., unpublished data). Therefore, we then examined the effect on cell viability in cisplatin-treated clear cell carcinoma cells and showed that knock-down of ADAM9m or inhibition of ADAM9m activity increases apoptotic cells, indicating enhancement of sensitivity to cisplatin in clear cell carcinoma cells. Similar effect of ADAM9 on resistance to chemotherapeutic drugs and radiation has been reported by siRNA-driven ADAM9 knock-down experiments in prostate carcinoma cells.[44] The authors in the study suggested that ADAM9 plays a role in therapeutic resistance by altering E-cadherin and integrin expression.[44] In colorectal carcinoma cell lines, chemotherapy-induced activation of ADAM17 is known to contribute to chemoresistance to 5-fluorouracil by transactivation of EGF receptor (EGFR) through shedding of EGFR ligands such as transforming growth factor-α.[45] Shedding of HB-EGF by ADAM17, ADAM9 and/or ADAM10 is also suggested to be related to resistance to doxorubicin in bladder and lung carcinoma cells.[46] In addition, a recent study on stomach carcinoma cell lines provided evidence that ADAM9 plays a role in EGFR transactivation through shedding of EGFR ligands.[47] In the present study, we provided data that EGFR activation is down-regulated by knock-down of ADAM9m expression. We also showed that ADAM9m exists on clear cell carcinoma cell membranes in an activated form and showed that knock-down of ADAM9m with shRNAs or inhibition of the activity with neutralizing anti-ADAM9m antibody enhances chemosensitivity to cisplatin and ADAM9m over-expression increases chemoresistance in ovarian clear cell carcinoma cells. Altogether, the data suggest that the activity of ADAM9m confers cisplatin resistance probably through the EGFR ligand shedding/EGFR activation axis, although further studies on the molecular mechanisms are necessary.

In conclusion, we have shown over-expression of ADAM9m in human ovarian clear cell carcinomas, and provided evidence that ADAM9m expressed on carcinoma cell membranes is involved in

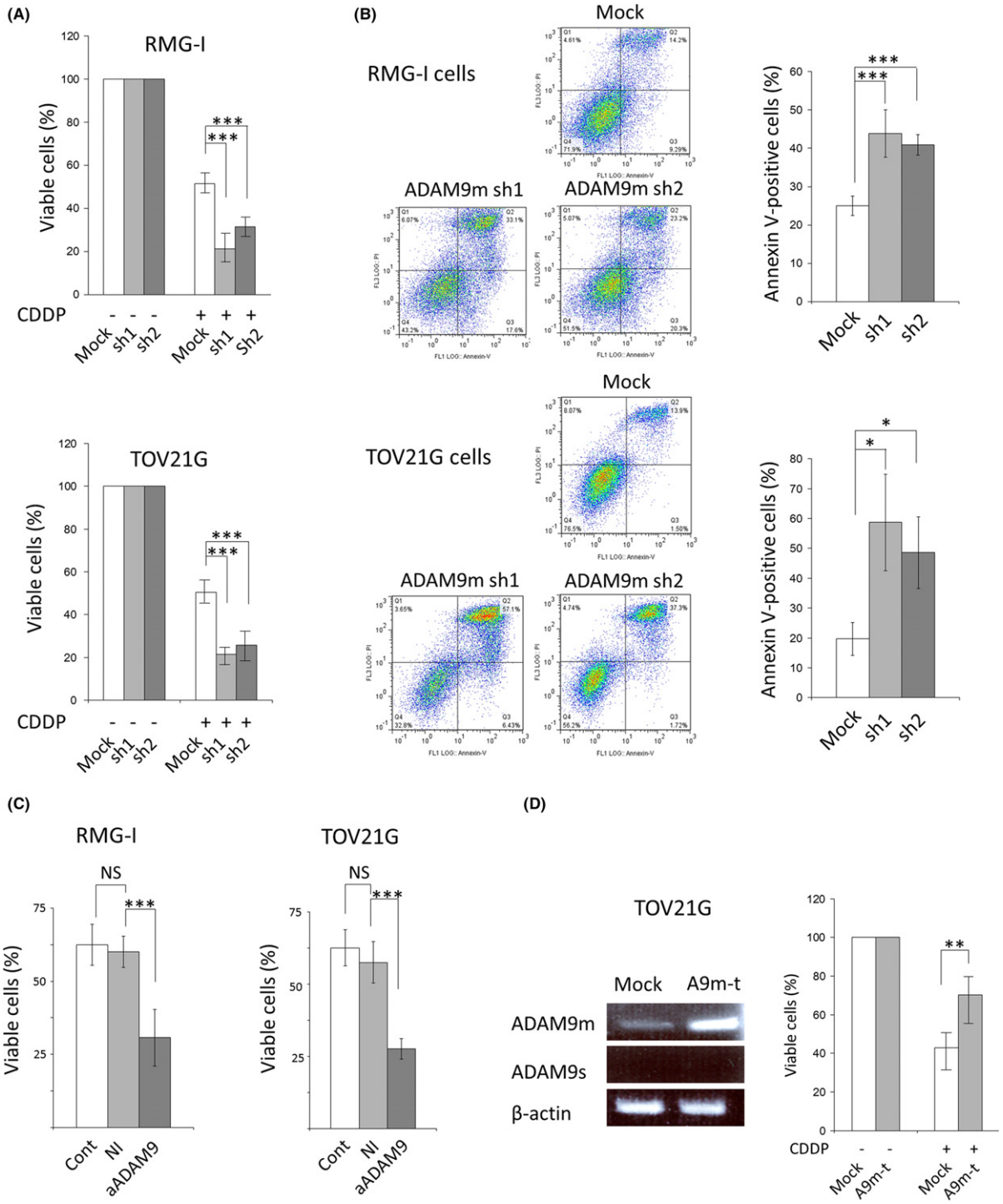

FIGURE 6 Effects of ADAM9m knock-down, inhibition of ADAM9m activity and ADAM9m over-expression on ovarian clear cell carcinoma cells after treatment with cisplatin. A, ADAM9m knock-down effect on cell viability after treatment with cisplatin. Viability of RMG-I and TOV21G cells treated without (CDDP−) or with cisplatin (CDDP+) was evaluated by MTT assay. B, Effect of ADAM9m knock-down on cisplatin-induced apoptotic cell death. Apoptotic cells showing positive reaction of propidium iodide (PI) and Annexin-V in Mock and ADAM9m shRNA transfectants (sh1 and sh2) were monitored by flow cytometric assay. C, Effect of neutralizing anti-ADAM9m antibody on cisplatin-treated cells. Viability of the cells treated with LD50 concentration of cisplatin in the presence of non-immune IgG (NI) or anti-ADAM9m antibody (αADAM9) was evaluated by MTT assay. Cont, the cells treated with LD50 cisplatin alone. D, ADAM9m over-expression effect on cisplatin-treated cells. mRNA expression and cell viability of TOV21G cells transfected with ADAM9m expression vectors (A9m-t) or mock vectors (Mock) were examined by RT-PCR and MTT assay, respectively. All assays were carried out in triplicate, and repeated 3 times. Bars, mean value ± SD. NS, not significant; *$P < .05$; **$P < .01$; ***$P < .001$. ADAM, a disintegrin and metalloproteinase

cisplatin resistance of clear cell carcinoma cells. These findings suggest that ADAM9m could be an attractive target for therapies of ovarian clear cell carcinomas when combined with established chemotherapeutics such as cisplatin.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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