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TROP-2 exhibits tumor suppressive functions in cervical cancer by dual inhibition of IGF-1R and ALK signaling

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HIGHLIGHTS

- *TROP-2* exhibits potent tumor suppressive functions in cervical cancer cells both *in vitro* and *in vivo*.
- *TROP-2* exerts its tumor suppressive effects by inhibiting the activity of IGF-1R and ALK receptors.
- TROP-2 sequesters the ligands of IGF-1R and ALK through protein interactions.

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ABSTRACT

Objective. Inactivation of tumor suppressor genes promotes initiation and progression of cervical cancer. This study aims to investigate the tumor suppressive effects of *TROP-2* in cervical cancer cells and to explain the underlying mechanisms.

Methods. The tumor suppressive functions of *TROP-2* in cervical cancer cells were examined by *in vitro* and *in vivo* tumorigenic functional assays. Downstream factors of *TROP-2* were screened using Human Phospho-Receptor Tyrosine Kinase Array. Small molecule inhibitors were applied to HeLa cells to test the *TROP-2* effects on the oncogenicity of IGF-1R and ALK. Protein interactions between TROP-2 and the ligands of IGF-1R and ALK were detected *via* immunoprecipitation assay and protein-protein affinity prediction.

Results. In vitro and *in vivo* functional assays showed that overexpression of *TROP-2* significantly inhibited the oncogenicity of cervical cancer cells; while knockdown of *TROP-2* exhibited opposite effects. Human Phospho-Receptor Tyrosine Kinase Array showed that the activity of IGF-1R and ALK was stimulated by *TROP-2* knockdown. Small molecule inhibitors AG1024 targeting IGF-1R and Crizotinib targeting ALK were treated to HeLa cells with and without *TROP-2* overexpression, and results from cell viability and migration assays indicated that the oncogenicity of vector-transfected cells was repressed to a greater extent by the inhibition of either IGF-1R or ALK than that of the *TROP-2*-overexpressed cells. Immunoprecipitation assay and protein-protein affinity prediction suggested protein interactions between TROP-2 and the ligands of IGF-1R and ALK.

Conclusions. Collectively, our results support that *TROP-2* exhibits tumor suppressor functions in cervical cancer through inhibiting the activity of IGF-1R and ALK.

1. Introduction

Cervical cancer is the fourth most common and lethal cancer among women [1]. A major risk factor of cervical cancer is human papillomaviruses (HPV) infection, which associates with over 90% of cervical cancer cases [2]. Accumulating evidence suggests that dysregulation of gene expression contributes to the pathogenesis of cervical cancer *via* activation of oncogenes and repression of tumor suppressor genes. Integration of the HPV genome to the host genome and the subsequent activation of the E6 and E7 oncoproteins contribute to the development

Abbreviations: TROP-2, trophoblast antigen 2; IGF-1R, insulin-like growth factor 1 receptor; ALK, anaplastic lymphoma kinase; XTT, 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide; RTK, Receptor Tyrosine Kinase.

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of cervical cancer [3]. Greenspan *et al.* reported that the Fragile Histidine Triad (*FHIT*) gene is frequently down-regulated in cervical cancer cell lines and clinical specimens [4]. The loss of *FHIT* has been linked with more aggressive tumor phenotypes and poor survival of cervical cancer patients [5]. Retinoic acid receptor beta, which has been found to be frequently suppressed in cervical cancer due to promoter hypermethylation, is also associated with cervical cancer progression [6].

Tumor-associated calcium signal transducer 2 (*TACSTD2* or *TROP-2*) is a cell surface glycoprotein with a large extracellular domain, a single transmembrane region and a short intracellular tail [7]. It was initially identified in human trophoblast and choriocarcinoma cell lines [8]. *TROP-2* has long been considered an oncogene. It is highly expressed on the cell surface of various types of cancer, including colorectal, pancreatic and ovarian carcinomas, and has been demonstrated to be capable of activating signaling pathways such as Akt, Jun and NF-κB signaling [8,9]. Interestingly, it has been reported that *TROP-2* was down-regulated in lung adenocarcinoma and head and neck squamous cell carcinoma with tumor suppressive effects [10,11]. Therefore, it would be necessary to further examine the functions of *TROP-2* and to understand its role in the development of different human tumors.

Insulin-like growth factor 1 receptor (IGF-1R) and anaplastic lymphoma kinase (ALK) are receptor tyrosine kinases that are reported to exert oncogenic effects in various cancers, including lung cancer and breast cancer for IGF-1R [12,13], and lymphoma and glioblastoma for ALK [14–16]. IGF-1R is the receptor for insulin-like growth factors IGF-1 and IGF-2, with higher affinity to IGF-1. Upon stimulation by IGF-1, IGF-1R functions to promote cell proliferation and survival. On the other hand, there are two ligands for ALK—pleiotrophin (PTN) and midkine (MDK) [16]. Activation of ALK could induce cell migration and survival in cancer [17]. Both IGF-1R and ALK have been reported to activate signaling pathways that drive cancer progression, including the Akt and Stat3 pathways [18,19].

Here, we report the potent tumor suppressive effects of *TROP-2* in cervical cancer cells. Human phospho-RTK array profiling results demonstrated the dual inhibition of IGF-1R and ALK by TROP-2. Inhibitors of IGF-1R (AG1024) and ALK (Crizotinib) exerts tumor suppressive effects in vector-transfected cells and were relatively ineffective in *TROP-2*-overexpressed cells. Immunoprecipitation experiments and protein-protein affinity prediction indicated physical bindings between TROP-2 and IGF-1, as well as between TROP-2 and MDK. We propose that TROP-2 might be able to compete with IGF-1R to bind IGF-1, and similarly with ALK to bind MDK. These competitive bindings provided a possible explanation of the inhibitory effects of TROP-2 on IGF-1R and ALK, the inhibition of which would lead to tumor suppression.

2. Materials and methods

2.1. Cell lines and reagents

HeLa cell lines were obtained from the Institute of Virology, Chinese Academy of Medical Sciences (Beijing, China). Cell line CaSki was kindly provided by Drs. Annie N.Y. Cheung (Department of Pathology, the University of Hong Kong) and Hextan Y.S. Ngan (Department of Obstetrics & Gynaecology, the University of Hong Kong). Both HeLa and CaSki cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS. Antibodies used in this study purchased from Cell Signaling Technology (Danvers, MA) include rabbit anti-ALK (Cat. number 3333; 1:1000 dilution in Western blotting), rabbit anti-p-ALK (Cat. number 3341; 1:1000 dilution in Western blotting), rabbit anti-p-IGF-1R (Cat. number 3024; 1:1000 dilution in Western blotting), rabbit anti-Akt (Cat. number 9272; 1:1000 dilution in Western blotting), rabbit anti-p-Akt (Ser473) (Cat. number 9271; 1:1000 dilution in Western blotting), rabbit anti-p-Akt (Thr308) (Cat. number 9275; 1:1000 dilution in Western blotting), rabbit anti-Stat3 (Cat. number 9132; 1:1000 dilution in Western blotting), rabbit anti-p-Stat3 (Ser727) (Cat. number 9134; 1:1000 dilution in Western blotting), rabbit anti-p-Stat3 (Tyr705) (Cat. number 9145; 1:1000 dilution in Western blotting) and rabbit anti-Flag (Cat. number 2368; 1:1000 dilution in Western blotting). Others include goat anti-TROP2 (Cat. number AF650; R&D Systems, Minneapolis, MN; 1:1000 dilution in Western blotting), rabbit anti-IGF-1R (Cat. number NB100-81980; Novus Biologicals, Littleton, CO; 1:1000 dilution in Western blotting) and mouse anti-β-Actin (Cat. number ab6276; Abcam, Cambridge, United Kingdom; 1:5000 dilution in Western blotting). Small molecule inhibitors AG1024 (Cat. number SC-205907) and Crizotinib (Cat. number SC-364471) were purchased from Santa Cruz Biotechnology (Dallas, TX).

2.2. TROP-2 overexpression and knockdown

For overexpression of *TROP-2* in HeLa cells, the 972 bp CDS region of *TROP-2* was amplified by PCR and cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Empty vector control and *TROP-2* expressing plasmids were transfected into HeLa cells using Lipofectamine 3000 (Invitrogen). 48 h after transfection, G-418 (Roche Diagnostics, Basel, Switzerland) was added to the cells to screen for stably transfected clones. For overexpression of *TROP-2* in CaSki cell line, cells were infected with lentivirus expressing *TROP-2*, followed by Blasticidin (Invitrogen) incubation to screen for stably transfected cells. Cell line transfected with pLenti6/V5 empty vector was used as control cell line. For knockdown of *TROP-2*, plasmids of two short hairpin RNAs targeting *TROP-2* mRNA and vector control plasmids were packaged into lentivirus and transduced into HeLa cell line. Puromycin (Sigma-Aldrich, St. Louis, MO) was used to screen for stably transfected cells. shRNA sequence lenti-sh2 was designed to target the sequence GTGATCACCAACCGGAGAAAG, while sequence lenti-sh4 was designed to target the sequence GAGAAAGGAACCGAGCTTGTA.

2.3. In vitro and in vivo oncogenicity function assays

For cell proliferation assay, 1000 cells/well were seeded in 96-well plates in triplicates and cell viability was measured using Cell Proliferation Kit II (Roche Diagnostics) for 5 or 6 days. For foci formation assay, 1000 or 2000 cells/well were seeded in 6-well plates in DMEM/10% FBS. After two weeks, colonies were stained with crystal violet and numbers of colonies were counted. In soft agar assay, 5000 cells/well were suspended in 0.35% agarose in DMEM/10% FBS in triplicates in 6-well plates as the upper layer, with 0.5% agarose in DMEM/10% FBS being the bottom layer. Numbers of colonies formed in each well were counted after 2 to 3 weeks. In migration and invasion assays, cells were suspended with serum-free DMEM medium and seeded in cell culture inserts or invasion chambers, with the bottom of the chambers immersed in DMEM/10% FBS. After 48 to 72 h, migrated or invaded cell were stained with crystal violet and counted under a light microscope. For *in vivo* tumor formation assay, cells were subcutaneously injected into the right and left dorsal flanks of nude mice. Tumor sizes were measured once per week. For *in vivo* cancer metastasis, cells were injected through the tail veins of severe combined immunodeficient (SCID) mice. Tumor metastatic nodules on the lungs were examined 8 weeks after injection. All animal procedures were approved by Committee on the Use of Live Animals in Teaching and Research, the University of Hong Kong.

2.4. Cell viability assay and TUNEL assay

For cell viability assay, 5000 cells/well were seeded in triplicates in 96-well plates and treated with cisplatin, AG1024 or Crizotinib for 24 h. Cell viability was measured with Cell Proliferation Kit II (XTT assay). The percentage of viable cells was calculated using the following formula: cell viability (%) = $A_{492/630}$ of treated cells/$A_{492/630}$ of control cells.

For TUNEL assay, cells were fixed with 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 (Sigma-Aldrich).

DNA of cells was labelled with TUNEL reaction mixture (Roche Diagnostics). Samples were then analyzed under a fluorescence microscope.

2.5. Human phospho-RTK array

Human phospho-RTK Array Kit (ARY001B, R&D Systems) allows for detection of 49 different phosphorylated RTKs. Protein lysates of *TROP-2* knockdown cells and control cells were incubated with membranes spotted with capture and control antibodies overnight at 4 °C, followed by incubation with an HRP-conjugated pan anti-phospho-tyrosine secondary antibodies for 2 h at room temperature. After incubating with ECL, signals on the membranes were visualized using X-ray films.

2.6. Immunoprecipitation assay

Cells were seeded in 100 mm dishes and were allowed to reach 60–80% confluency. After transient transfection, cells were lysed with RIPA and cell lysates were incubated with anti-Flag affinity gel (Sigma-Aldrich) or anti-V5 affinity gel (Sigma-Aldrich) overnight at 4 °C, followed by collection and washing of resin beads. 30 μL of 2 × SDS sample buffer was added to the beads and boiled for 15 min to elute the bound proteins.

2.7. Western blotting

Protein samples were denatured and separated in SDS-PAGE gel and transferred to PVDF membranes. The membranes were subsequently blocked with 5% non-fat milk and incubated with primary antibodies at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibodies for 1 h at room temperature and incubation with ECL for 1 min. X-ray films were used to visualize the chemiluminescent signals on the membranes.

2.8. Statistical analyses

Student's *t*-test was applied to compare the mean values of two groups using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). Error bars represent standard deviation values. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. TROP-2 overexpression inhibits oncogenicity of cervical cancer cells

To examine the effects of *TROP-2* on the oncogenicity of cervical cancer cells, the coding region of this gene was cloned into pcDNA3.1 vector and transfected into HeLa cells. Two *TROP-2*-overexpressing clones with the highest expression levels of *TROP-2*, namely Clone 8 (C8) and Clone 9 (C9), were selected for functional analyses. Empty vector-transfected cells were used as controls. Western blot analysis confirmed the overexpression of *TROP-2* in both clones (Fig. 1A).

Cell proliferation of vector- and *TROP-2*-transfected cells were examined by XTT assay. Results of XTT assay showed that overexpression of *TROP-2* significantly inhibited cell growth of both C8 and C9 clones of HeLa cells ($P < 0.05$; Fig. 1B). Foci formation assay and soft agar assay showed that both anchorage-dependent and anchorage-independent colony formation capabilities of HeLa cells were significantly suppressed in *TROP-2*-transfected clones when compared to vector-transfected cells ($P < 0.001$; Fig. 1C). The *in vitro* cell migration and invasion capabilities of *TROP-2*-transfected clones were also significantly inhibited ($P < 0.01$; Fig. 1D).

The functions of *TROP-2* in *in vivo* tumorigenicity were studied *via* two assays: *in vivo* tumor formation assay and tail vein injection assay. For *TROP-2*-overexpressed cells, the C9 clone of HeLa cells was used in these two assays as *TROP-2*-overexpressed cells because of its slower cell growth than the other clone (C8). For *in vivo* tumor formation assay, *TROP-2*-overexpressed and control cells (7 million cells per cell line) were injected subcutaneously at the right and left dorsal flanks of each one of the nude mice ($n = 5$ in each group), respectively. Tumor sizes were measured once a week for 5 weeks. Tumor growth results demonstrated that the tumor formation capability of *TROP-2*-overexpressing cells was significantly inhibited when compared to control cells ($P < 0.001$; Fig. 1E). To test the effect of *TROP-2* on tumor metastasis *in vivo*, 0.7 million cells (1/10 of cells used in tumor formation assay) of either HeLa-Vec or HeLa-*TROP2* were injected through the tail veils of SCID mice ($n = 5$ in each group). After 2 months, the mice were sacrificed and the visible metastatic nodules in the lungs were examined. It has been shown that HeLa-Vec cells were able to form large tumor nodules in 3 of the 5 mice, while HeLa-*TROP2* cells could not form visible tumor nodules in any of the mice tested (Fig. 1F). The tumor suppressive functions of *TROP-2* in cervical cancer cells had been further confirmed using two additional *TROP-2*-overexpressing clones of HeLa (C15 and C31) and an additional cervical cancer cell line CaSki (Supplementary Fig. 1).

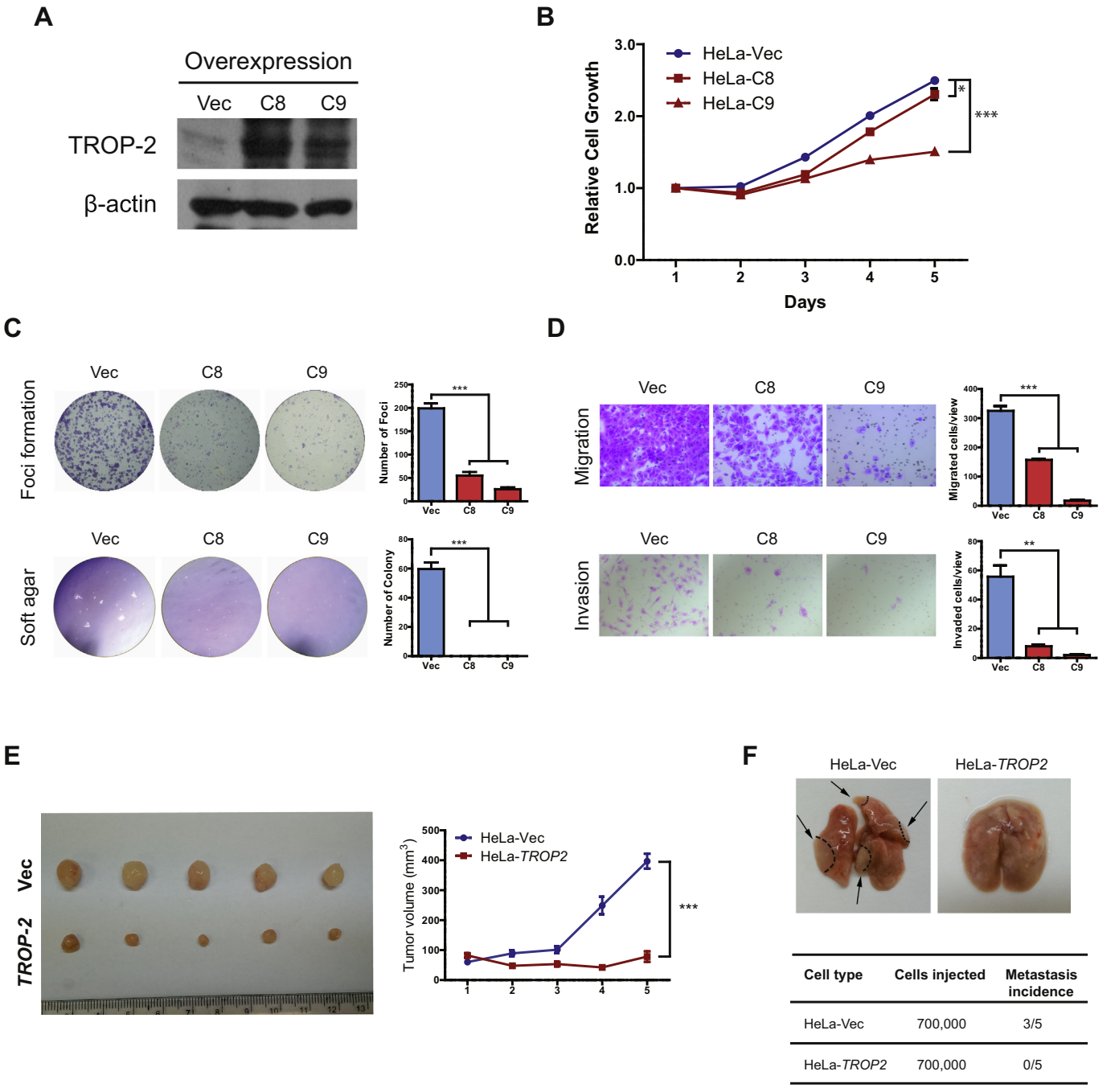
3.2. Knockdown of TROP-2 promotes oncogenicity of HeLa cells

Upon demonstration of the tumor suppressive effects of *TROP-2* overexpression, the expression of this gene was further knockdown in HeLa cells. Two shRNA sequences (sh2 and sh4) targeting the *TROP-2* mRNA were cloned and stably transfected into HeLa cells. Empty vector-transfected cells were used as controls. Western blotting confirmed the knockdown of *TROP-2* using both shRNAs (Fig. 2A). For cell proliferation, XTT assay was used to compare cell growth rates between *TROP-2* knockdown cells and control cells, and the result showed that *TROP-2* knockdown significantly accelerated cell proliferation of HeLa cells ($P < 0.001$; Fig. 2B). *TROP-2* knockdown cells also showed significantly increased numbers of colonies formed in foci formation and soft agar assays when compared to the control cells ($P < 0.05$; Fig. 2C). In addition, knockdown of *TROP-2* greatly promoted *in vitro* cell migration and invasion capabilities of HeLa cells ($P < 0.01$; Fig. 2D).

For *in vivo* tumor formation experiment, HeLa-sh2 cells were randomly selected for injection as HeLa-sh2 and HeLa-sh4 cells demonstrated similar cell growth rates. 5 million cells of HeLa-vec and HeLa-sh2 were injected subcutaneously at the left and right dorsal flanks of nude mice ($n = 5$ in each group), respectively. Tumor growth curves were generated *via* weekly measurement of tumor sizes for 3 consecutive weeks. It was shown that the tumor growth capability of HeLa cells was significantly enhanced by knockdown of *TROP-2* ($P < 0.05$; Fig. 2E). In summary, the above results demonstrated that *TROP-2* knockdown could significantly promote oncogenicity of cervical cancer cells both *in vitro* and *in vivo*.

3.3. TROP-2 sensitizes cervical cancer cells to cisplatin-induced apoptosis

To test the effect of *TROP-2* on the chemosensitivity of cervical cancer cells, *TROP-2*-overexpressing and knockdown HeLa cells along with the respective control cells were incubated with cisplatin for 24 h, followed by XTT assay for cell viability measurement. To examine the effects of *TROP-2* overexpression, *TROP-2*-expressing and empty vector plasmids were transiently transfected to HeLa cells using Lipofectamine 3000. Gradient concentrations of cisplatin was added to the cells 48 h post-transfection. XTT assay was performed after 24 h of incubation with cisplatin (72 h post-transfection). Cell viabilities of vehicle treated groups were normalized to 1; cell viabilities in the cisplatin treated groups were normalized to that of the vehicle treated groups of the same cell line. Results showed that *TROP-2*-transfected cells were more sensitive to cisplatin-induced cell apoptosis in a dose-dependent manner. At low concentrations of cisplatin treatment (*i.e.*, 2 μg/mL, 4 μg/mL and 6 μg/mL), there was no significant difference in cell viability between vector and *TROP-2*-transfected cells. At higher cisplatin concentrations, *TROP-2*-transfected cells showed significantly



Cell type	Cells injected	Metastasis incidence
HeLa-Vec	700,000	3/5
HeLa-*TROP2*	700,000	0/5

Fig. 1. *TROP-2* exhibits potent tumor suppressive functions in HeLa cells. (A) Western blotting showing overexpression of *TROP-2* in HeLa cells. β-actin was used as loading control. (B) XTT assay detecting cell proliferation of vector control cells and *TROP-2*-overexpressing clones of HeLa cells. $^{*}P < 0.05$, $^{***}P < 0.001$, Student's *t*-test. (C) Foci formation and soft agar assays of vector control and *TROP-2*-overexpressing clones of HeLa cells. $^{***}P < 0.001$, Student's *t*-test. (D) Migration and invasion assays of vector control and *TROP-2*-overexpressed clones of HeLa. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test. (E) *In vivo* tumor formation assay of *TROP-2*-overexpressed and control cells of HeLa. $^{***}P < 0.001$, Student's *t*-test. (F) Lung metastasis of HeLa-Vec and HeLa-*TROP2* (C9) cells injected through tail vain in SCID mice. Arrow heads indicate positions of tumor nodules; dotted lines indicate tumor nodule areas.

lower cell viability when compared to vector-transfected cells ($P < 0.05$; Fig. 3A). On the other hand, XTT assay showed that *TROP-2* knockdown cells are more resistant to the treatment of cisplatin when compared to vector-transfected cells, as indicated by the higher cell viability of *TROP-2* knockdown cells than that of the control cells ($P < 0.05$; Fig. 3B).

Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay on vector-transfected and *TROP-2*-overexpressing clones of HeLa cells further verified the above findings. Green labelling indicates DNA double-stranded breaks and thus cells undergoing apoptosis, while blue labelling represents cell nucleus. As shown in Fig. 3C, cisplatin treatment induced significantly more DNA breaks in *TROP-2*-overexpressing clones than in control cells ($P < 0.001$).

The effect of *TROP-2* on modulating the activity of apoptotic marker PARP was investigated *via* Western blot analysis. Results showed that the cleaved (activated) form of PARP was elevated to a greater extent in *TROP-2* transient transfected HeLa cells when compared to vector control cells upon cisplatin treatment, suggesting that *TROP-2* sensitizes cancer cells to cisplatin-induced apoptosis (Fig. 3D).

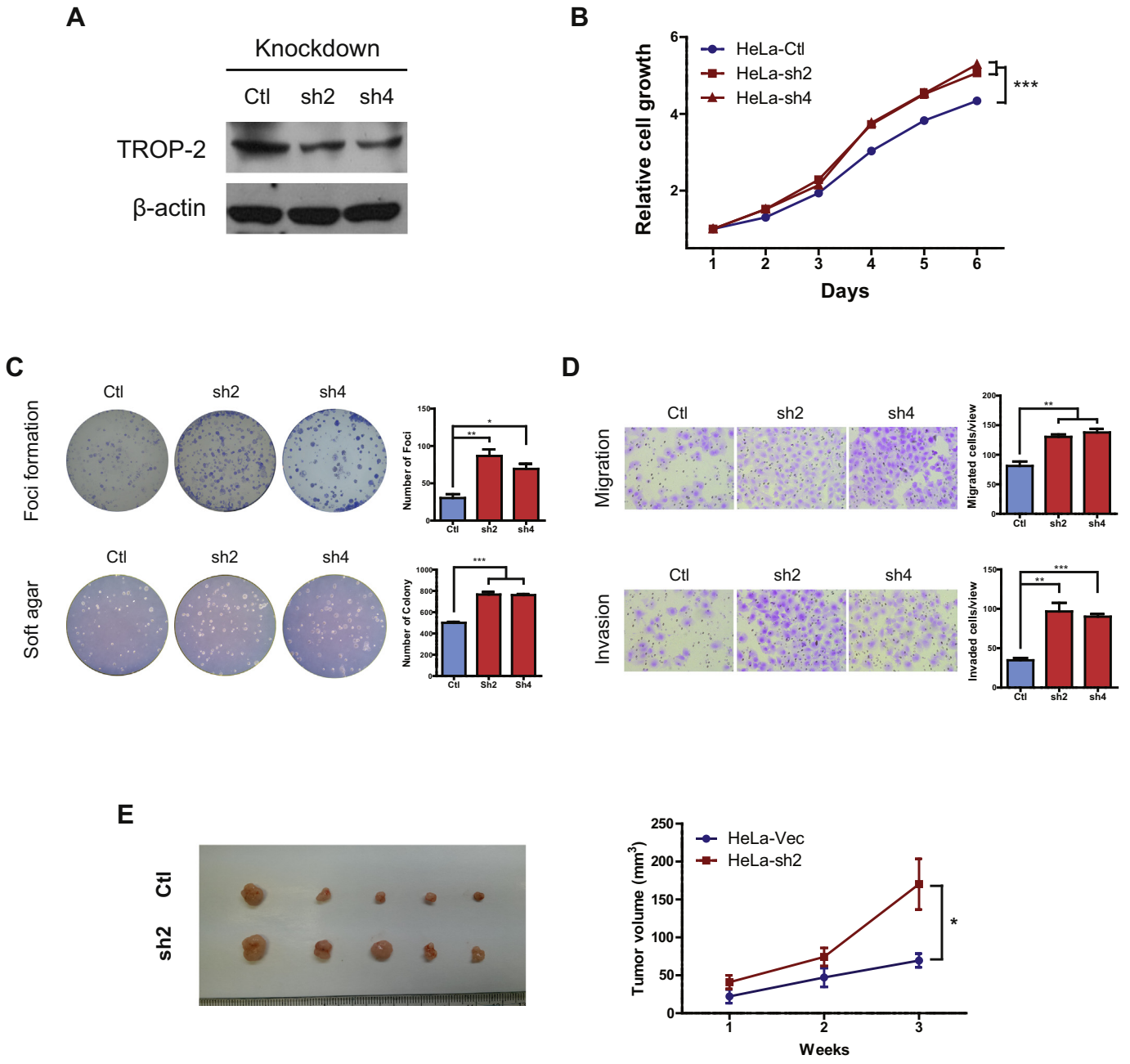


Fig. 2. Knockdown of *TROP-2* promotes oncogenicity of HeLa cells. (A) Western blotting showing knockdown of *TROP-2* in HeLa cells. β-actin was used as loading control. (B) XTT assay detecting cell proliferation of vector control and *TROP-2* knockdown cells of HeLa. $^{***}P < 0.001$, Student's *t*-test. (C) Foci formation and soft agar assays of vector control and *TROP-2* knockdown cells of HeLa. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test. (D) Migration and invasion assays of vector control and *TROP-2* knockdown cells of HeLa. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test. (E) *In vivo* tumor formation assay of *TROP-2* knockdown and control cells of HeLa ($n = 5$). $^{*}P < 0.05$, Student's *t*-test.

3.4. Down-regulation of TROP-2 activates IGF-1R and ALK signaling pathways

Accumulating evidence indicates that hyperactivation of human RTKs greatly contribute to cancer progression [20,21]. To dissect the downstream signaling of *TROP-2* in cervical cancer, human phospho-RTK array was performed on HeLa-sh2 and control cells. This assay detects the activity of 49 different human RTKs simultaneously. As indicated by the signal intensities, both IGF-1R and ALK showed higher activity in HeLa-sh2 cells when compared with control cells (Fig. 4A). This result was further verified by Western blotting, which showed that both p-IGF-1R and p-ALK were up-regulated in *TROP-2* knockdown cells and suppressed in *TROP-2* transient transfected cells of HeLa when compared to the respective control cells (Fig. 4B). Since AKT and STAT3 are common downstream targets of IGF-1R and ALK [18,19], their levels of activation were also examined. As expected, both AKT and STAT3 pathways were activated in *TROP-2* knockdown cells and inhibited in *TROP-2* transient transfected cells when compared to respective control cells (Fig. 4B).

3.5. TROP-2 interacts with IGF-1 and MDK

IGF-1 and MDK are ligands of IGF-1R and ALK, respectively. IGF-1R and ALK will be activated upon binding to these two factors. Given that TROP-2 is located on the cell membrane and possesses a large extracellular domain with a putative epidermal growth factor-like (EGF) domain [7], we hypothesize that TROP-2 might competitively bind to the ligands of IGF-1R and ALK and subsequently suppress the activation

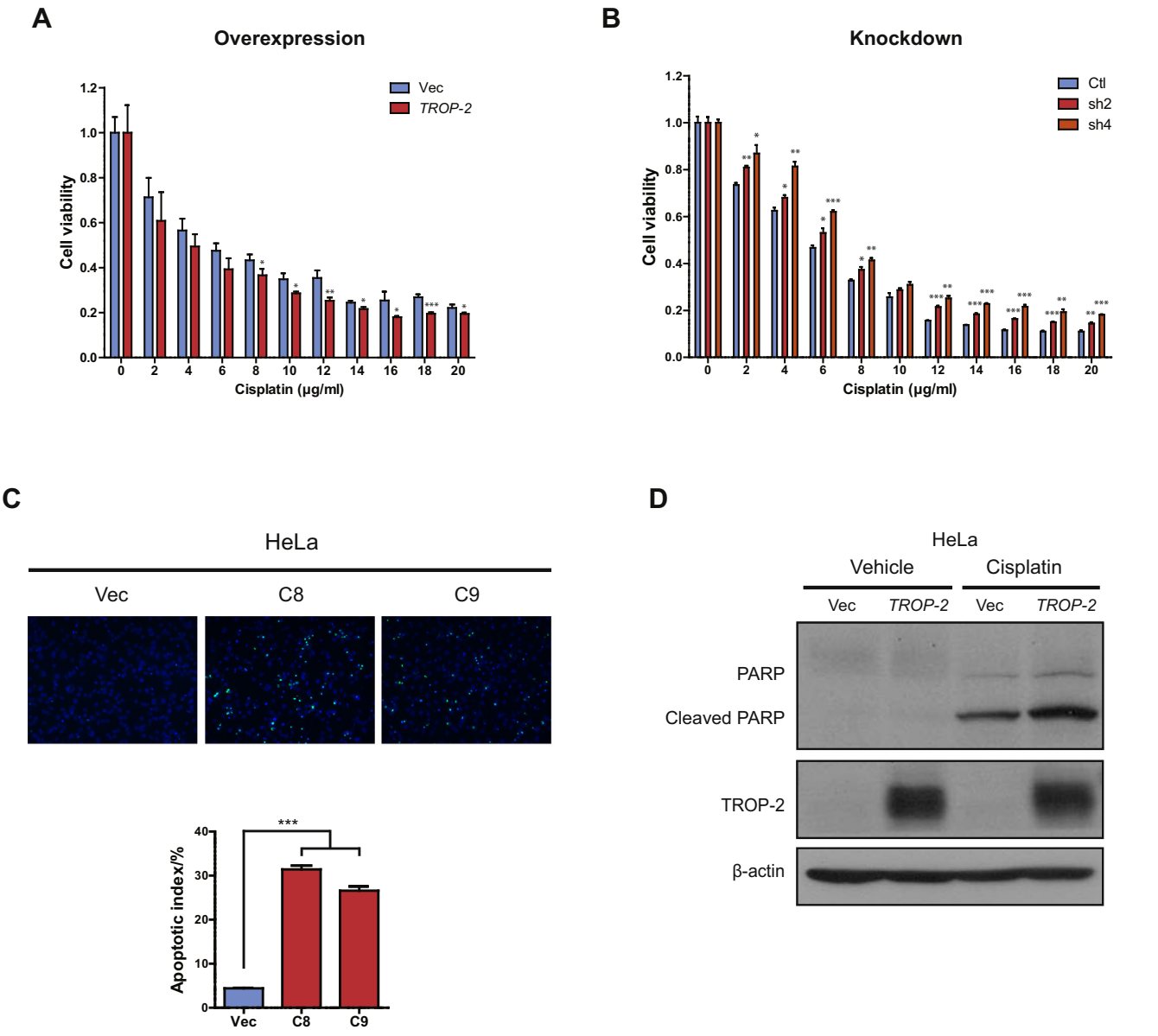


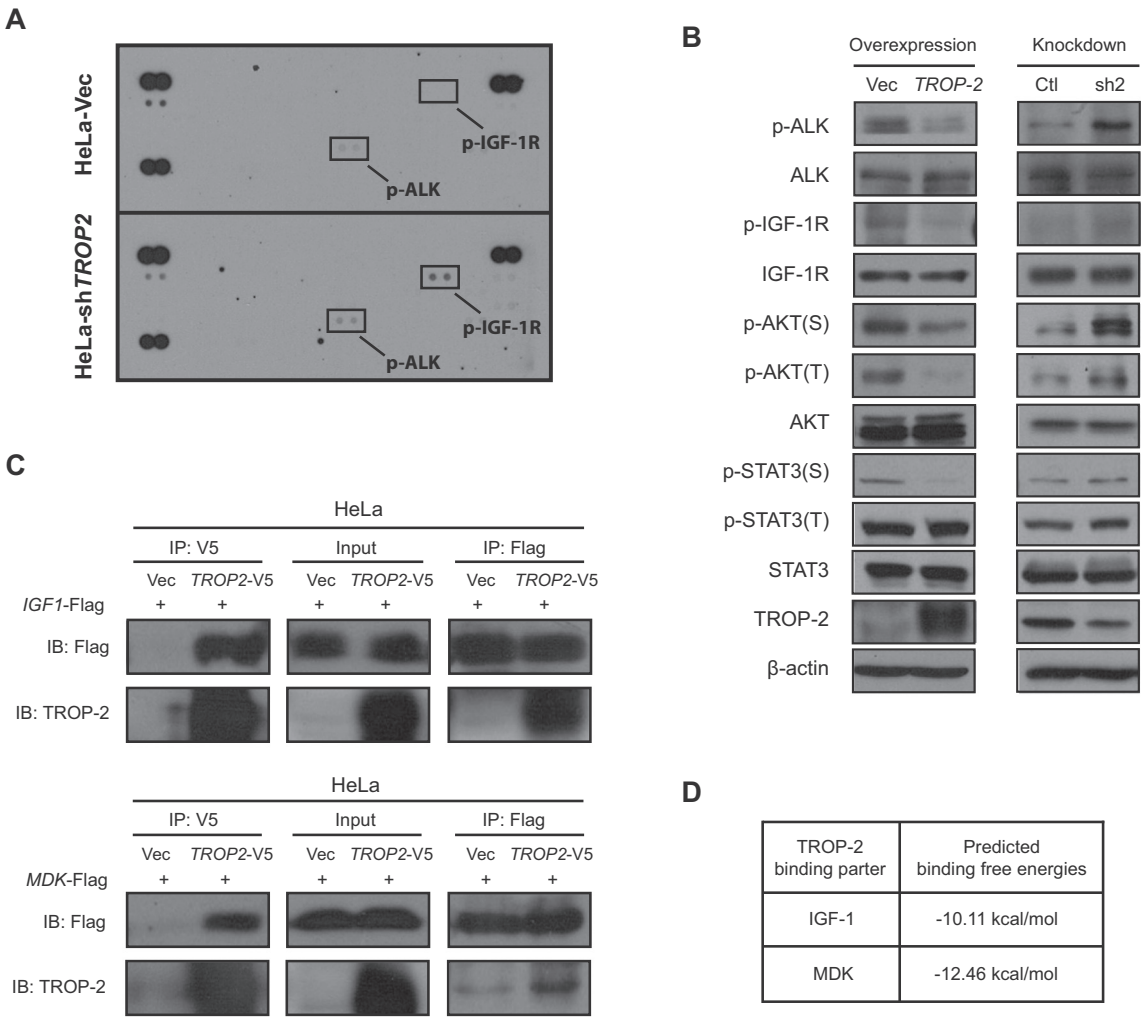
Fig. 3. *TROP-2* promotes cisplatin-induced cell apoptosis. (A) Cell viability assay on *TROP-2*-overexpressed and control cells of HeLa after treatment of gradient concentrations of cisplatin. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test. (B) Cell viability assay on *TROP-2* knockdown and control cells of HeLa after treatment of gradient concentrations of cisplatin. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test. (C) TUNEL assay on *TROP-2*-overexpressed and control cells of HeLa after cisplatin treatment (8 μg/ml, 24 h). $^{***}P < 0.001$, Student's *t*-test. (D) Western blot analysis of (cleaved) PARP with or without cisplatin treatment in HeLa-Vec and HeLa-*TROP2* cells. β-actin was used as loading control.

of these two receptors. To test this hypothesis, *IGF1* and *MDK* (both Flag-tagged) were cloned into pcDNA3.1 vector and co-transfected with *TROP-2* (V5-tagged) into HeLa cells. 48 h after transfection, immunoprecipitation followed by Western blotting was performed to detect interactions between TROP-2 and IGF-1 or MDK. Our data showed that when IGF-1 or MDK was pulled down by anti-Flag gel, TROP-2 binding was detected in both IGF-1 and MDK transfected cells (Fig. 4C). This result has been confirmed by pull-down using anti-V5 gel, where TROP2-V5 was pulled down with IGF-1 and MDK (Fig. 4C). These data indicate that the TROP-2 protein is in the same protein complexes as IGF-1 and MDK.

Provided that TROP-2 could form protein complexes with IGF-1 and MDK, we further explored the possibility of TROP-2 directly binding to IGF-1 and MDK in these protein complexes. To this end, a prediction of protein binding affinity between TROP-2 and IGF-1 or MDK was performed using an online tool–PPA-Pred2 (Protein-Protein Affinity Predictor). This tool provides the binding free energy between two proteins based on their protein sequences. A negative binding free energy value indicates spontaneous binding between two proteins; and the higher the absolute value of binding free energy, the more stable the binding [22]. The binding free energies were predicted to be −10.11 kcal/mol between TROP-2 and IGF-1 and −12.46 kcal/mol between TROP-2 and MDK under the "Receptor containing" category (Fig. 4D). The absolute values of these two binding free energies fell within the range of typical receptor-ligand binding free energies of an average absolute value of 11.58 kcal/mol [23]. These data of protein-protein affinity prediction support that TROP-2 might be capable of directly interacting with IGF-1 and MDK.

3.6. TROP-2-overexpressed cells are relatively irresponsive to the inhibition of IGF-1R and ALK

To elucidate the functions of IGF-1R and ALK in cervical cancer, AG1024 and Crizotinib were used to inhibit the activity of IGF-1R and ALK, respectively. The efficacy of these two drugs were first verified by Western blot analysis on HeLa cells after treatment of these drugs for 24 h. Treatment of AG1024 inhibited the activity of IGF-1R and its



TROP-2 binding parter	Predicted binding free energies
IGF-1	-10.11 kcal/mol
MDK	-12.46 kcal/mol

Fig. 4. *TROP-2* down-regulation activates IGF-1R and ALK signaling pathways. (A) Human phospho-RTK array of HeLa-Vec and HeLa-sh2 cell lysates. Signal intensities at the same locations of two membranes indicate the expression levels of the same phospho-RTK in the two samples. (B) Confirmation of phospho-RTK array results using Western blotting. (p-)IGF-1R, (p-)ALK, (p-)AKT and (p-)STAT3 were detected on *TROP-2*-overexpressed and knockdown cells and the respective control cells of HeLa. β-actin was used as loading control. (C) Vector and *TROP-2* expressing plasmids were transiently co-transfected with IGF-1 or MDK in HeLa cells. Immunoprecipitation and Western blotting were applied to detect the interactions between TROP-2 and IGF-1 or MDK. (D) The predicted binding free energies between TROP-2 and IGF-1 and between TROP-2 and MDK using PPA-Pred2 (Protein-Protein Affinity Predictor).

down-stream effector AKT, while treatment of Crizotinib inhibited the activity of ALK and the downstream factor AKT (Supplementary Fig. 2).

Next, we would like to study the effects of *TROP-2* on cell death induced by AG1024 and Crizotinib treatments in cervical cancer cells. Vector and *TROP-2*-expressing plasmids were transiently transfected into HeLa cell line. 48 h after transfection, *TROP-2* or vector transfected cells were incubated with gradient doses of AG1024 or Crizotinib. After 24 h of incubation, cell viabilities were examined by XTT assay. The cell viabilities of vehicle treated cells in both vector-transfected and *TROP-2*-transfected groups were normalized to 100%. The cell viabilities of AG1024 and Crizotinb treated cells were normalized to the respective vehicle treated groups and represented in percentages to those of the vehicle treated groups. Results showed that *TROP-2*-overexpressed cells sustained higher percentage of cell viabilities than vector-transfected cells upon AG1024 or Crizotinib treatments, as shown by the significantly higher cell viability in *TROP-2*-transfected cells than that of the vector control cells ($P < 0.05$; Fig. 5A & B).

The inhibitory effects of AG1024 and Crizotinib on cell migration were also compared between vector- and *TROP-2*-transfected HeLa cells. In accordance with the results from cell viability assay as described above, the numbers of migrated cells of vehicle treated groups in both vector-transfected and *TROP-2*-transfected groups were normalized to 100%. The numbers of migrated cells after AG1024 and Crizotinb treatment were normalized to the respective vehicle treated groups and represented in percentages to those of the vehicle treated groups. Results of the migration assay showed that AG1024 and Crizotinib treatments inhibited cell migration to a greater extent in vector-transfected cells than in *TROP-2*-transfected cells ($P < 0.001$; Fig. 5C).

4. Discussion

Dysregulation of oncogenes and tumor suppressor genes greatly contributes to cancer onset and progression; much attention has been attracted to investigate the functions of these important genes in cancer. *TROP-2* has long been considered an oncogene, which exhibits strong tumorigenic features in different cancer types, such as prostate [24], breast [25] and colon [26] carcinomas. On the other hand, it has also been reported that *TROP-2* is frequently lost and exhibits tumor suppressive functions in head and neck cancer and lung cancer [10,11]. In a report by Wang *et al.*, *TROP-2* knockout promoted epithelial-mesenchymal transition of keratinocytes and skin tumor formation in $ARF^{-/-}$ C57BL/6 mice. Immortalized keratinocyte cell line derived from $TROP\text{-}2^{-/-}ARF^{-/-}$ mice demonstrated enhanced capabilities of cell proliferation and migration [27]. Therefore, the exact function of this gene in the regulation of cancer progression might be organ and cancer type dependent.

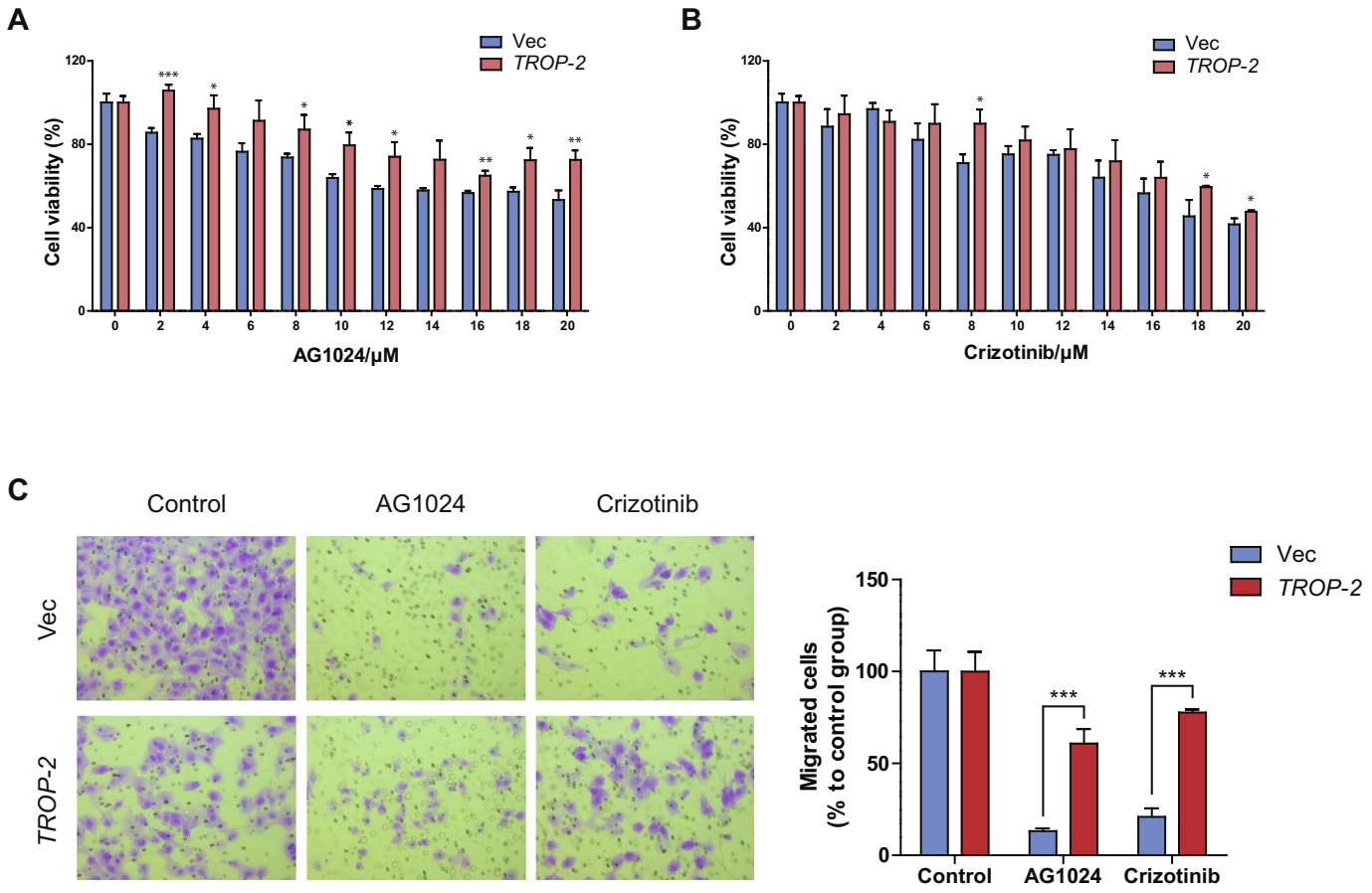


Fig. 5. *TROP-2*-overexpressed cells are relatively irresponsive to the inhibition of IGF-1R and ALK. (A) Comparison of cell viability between vector- and *TROP-2*-transfected HeLa cells after AG1024 treatment for 24 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test. (B) Comparison of cell viability between vector- and *TROP-2*-transfected HeLa cells after Crizotinib treatment for 24 h. $^{*}P < 0.05$, Student's *t*-test. (C) Comparison of cell migration capabilities between vector- and *TROP-2*-transfected HeLa cells when incubated with AG1024 or Crizotinib for 48 h. $^{***}P < 0.001$, Student's *t*-test.

As to the expression level of *TROP-2* in cervical cancer, several groups have reported overexpression of TROP-2 protein in tumor tissues. The overexpression of TROP-2 protein correlates with more aggressive phenotypes of cervical cancer specimens and poor prognosis of cervical cancer patients [28–30]. In this report, *TROP-2* demonstrates potent tumor suppressive effects in cervical cancer cells. The tumor suppressive functions of *TROP-2* in cervical cancer we report herein seem to contradict with the predominant overexpression of this gene in cervical cancer as reported previously. However, there are evidence hinting that overexpression of a gene in tumor would not necessarily exclude the possibility of this gene being a tumor suppressor. There are two possible explanations. Firstly, there are examples of tumor suppressor genes being up-regulated in cancer. A representative example is the well-known tumor suppressor *TP53* gene, which encodes the p53 protein. Weiss *et al.* reported frequent overexpression of p53 in skin cancer cell lines and clinical specimens [31]. Zhang *et al.* showed hyperactivation of p53 protein in the nucleus of bladder cancer cells [32]. It was later comprehensively reported that the *TP53* gene is frequently mutated in over 50% of cancer types and the missense and nonsense mutations of this gene greatly contribute to cancer progression [33–35]. What happens in *TP53* might also apply to the *TROP-2* gene. Possibly, it is not the loss of expression of *TROP-2*, but the mutation of this gene is contributing to the initiation and progression of cervical cancer. Secondly, it has been established that under certain circumstances, the maintenance and survival of cancer cells are dependent upon the expression of non-oncogenes. Such phenomenon has been proposed as the "non-oncogene addiction" model. A well-studied example for this model is the gene heat shock factor 1 (*HSF1*), which is a critical modifier in heat shock response and plays significant roles in promoting cell survival under various physiological stresses. Being important for normal cell functions, *HSF1* has been found to support oncogenic transformation in both marine and human cancer models by maintaining cell proliferation and survival [36]. Similarly, for cervical cancer patients with up-regulated *TROP-2* level, the cancer cell functions might be dependent on the non-oncogenic *TROP-2* expression.

Receptor tyrosine kinases are membrane receptors that are bound and activated by hormones, cytokines or growth factors in a paracrine or autocrine manner. These receptors control a plethora of intracellular signaling pathways mediating a wide range of cellular functions, such as cell proliferation, differentiation and apoptosis [37]. It has been well established that constitutive activation of RTKs and aberrant RTK signaling drive cancer progression. Many RTKs act as oncogenes involving in most forms of human malignancies through activating oncogenic signaling pathways including Akt and Stat3 signaling [38]. Examples include HER2, a tyrosine receptor with low expression in normal epithelial cells found to be significantly elevated in various types of cancer including breast, ovarian, gastric, lung and bladder carcinomas [37]. In this study, *TROP-2* has been found to inhibit the activation of IGF-1R and ALK. The EGF-like extracellular domain of TROP-2 protein facilitates the binding between TROP-2 and candidate growth factors [7]. In the case of cervical cancer, we propose that TROP-2 probably binds to IGF-1 and MDK as indicated by the results from immunoprecipitation and protein affinity prediction (Fig. 4). Therefore, a loss-of-function mutation of TROP-2 on the extracellular domain could possibly render this protein no longer capable of binding IGF-1 and MDK, leading to hyperactivation of IGF-1R and ALK and subsequently promoting oncogenicity.

In summary, we report herein that *TROP-2* demonstrates tumor suppressive functions in cervical cancer cells by dual inhibition of IGF-1R and ALK. Although it has been reported previously that TROP-2 protein expression is predominantly up-regulated in cervical cancer tissues, potential loss-of-function mutations in the *TROP-2* gene and possible "non-oncogene addiction" mechanisms of cancer cells might provide the missing link between the overexpression of *TROP-2* and the tumor suppressive effects of this gene in cervical cancer. The evidence presented in this study also indicates that IGF-1R and ALK are potential drug targets in future targeted therapy for cervical cancer patients.

Conflict of interests

The authors declare no conflict of interests.

Author contributions

S. Sin: designed and performed experiments; Y. Li and M. Liu: designed experiments; S. Ma and X. Guan: experiment design, resources support and supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2018.10.039.

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