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Trophinin is a potent prognostic marker of ovarian cancer involved in platinum sensitivity

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Abstract

Ovarian cancer is the leading cause of death in women with gynecological malignancies, with prognosis of advanced stage tumors determined by chemotherapeutic response and the success of tumor resection. Since aberrant RAS pathway activation is frequent in ovarian cancer, study of *in vitro* RAS-induced transformation and accompanying genomic expression changes in ovarian surface epithelial cells is imperative for development of new therapeutic modalities and for understanding tumorigenesis. cDNA microarray analysis revealed TROPHONIN (TRO), a homophilic adhesion molecule involved in blastocyst implantation, was among the genes most down-regulated by RAS induction. TRO expression is higher in cisplatin-sensitive cancer cell lines and positively correlates with prognoses in ovarian cancers. TRO knockdown by RNA interference conferred cisplatin resistance and led to increased invasiveness of cultured ovarian cancer cells. These findings underscore the importance of TRO in tumorigenesis, and suggest that TRO may be a useful biomarker for cisplatin sensitivity and invasive potential.
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Ovarian cancer is the leading cause of death from malignant gynecological tumors with 15,310 deaths in the United States in 2006 [1]. Most cases of ovarian cancer are diagnosed at an advanced stage, with gross tumor growth in omentum and the abdominal cavity, features that carry a higher relative risk than age or histology in multivariate analysis [2]. The overall response rates for chemotherapy using platinum and paclitaxel range from 70% to 85%, but 50% to 70% of responders acquire drug resistance during chemotherapy and relapse [3]. In the platinum resistant setting, other agents produce a poor response rate (7–35%) [4]. Thus, platinum sensitivity along with optimal tumor resection largely determines prognosis.

During tumorigenesis, multiple signaling pathways, including RAS, SRC, and MYC, are deregulated through accumulation of genetic and epigenetic alterations in the genome. Mutations in *KRAS* have been linked to the mucinous and serous histologic types of ovarian tumors [7]. Furthermore, activation of the RAS/RAF-signaling pathway without detectable RAS mutation is common in high-grade ovarian cancer [5,8], presumably due to deregulation of upstream signaling molecules Her-2/Neu [9] and Her-3 [10]. Oncogenic RAS, has the ability to transform

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cells *in vitro* [6] and thus RAS pathway deregulation has been used as a fundamental tool for the study of tumorigenesis. We previously established a method for *in vitro* transformation of primary ovarian surface epithelial cells (OSE), through immortalization by SV40 LT antigen and hTERT, and transformation by subsequent introduction of a constitutively active form of RAS or ERBB2 [11]. *In vitro* RAS-transformed human ovarian cancer cells share features with ovarian cancer cells *in vivo*, including histology and ability to produce ascites [7]. RAS pathway activation confers oncogenic properties to cells along with resistance to platinum drugs [12] although the molecular mechanisms remain to be elucidated.

In ovarian cancer, acquisition of invasiveness is often accompanied by epithelial–mesenchymal transition (EMT) [13], a phenomenon necessary for development and implantation. EMT involves commitment to a fibroblastic cell type with drastic alterations in cytoskeleton and cell-membrane components [14]. Numerous signaling pathways mediate EMT resulting in the loss of E-cadherin [13,14]. RAS-RAF signaling also plays an important role in EMT during tumor progression [15].

To understand the consequence of RAS activation in OSE cells, we used cDNA microarrays to identify genes that are induced or suppressed by RAS [11]. We previously surveyed genes upregulated by RAS and reported that Neuropilin-1 plays an important role in loss of contact inhibition of cancer cells [16]. Herein we investigated genes downregulated by RAS to identify molecule(s) potentially protective against RAS-mediated transformation. We focused on trophinin (TRO), a MAGE (melanoma-associated antigen)-D family [17] membrane protein originally isolated as a cell adhesion molecule mediating attachment between human trophoblasts and endometrial cells [18]. Although TRO or MAGE-D1 have functional roles in other cancers [19–21], their role in ovarian cancer is presently unclear.

Here we describe the relationship between ovarian cancer phenotypes and TRO. TRO expression correlates with better prognosis, and knockdown by RNAi enhances cisplatin resistance and invasiveness of cultured ovarian cancer cells. These observations indicate an importance role for TRO in ovarian tumorigenesis, and suggest its usefulness as a biomarker for cisplatin sensitivity and invasiveness.

Materials and methods

Microarray analysis of RAS-transformed cells. cDNA microarray analysis was conducted as described previously [22] using immortalized OSE cells with or without RAS [11].

Tissue collection. Ovarian cancer surgical specimens were obtained with written consent and were treated for histological analysis as previously described [16] with approval from the Institutional Review Board at Kyoto University.

Cell lines, cell culture, and in vitro assays. Human ovarian cancer cell lines OVCAR-3, SK-OV-3, A2780-s, and A2780-cp (ATCC; Rockville, MD), TYK-nu and TYK-nu CP-r (Japan Health Sciences Foundation; Tokyo, Japan), CH1 (The Institute of Cancer Research tissue culture bank) and MCAS (The Japanese Cancer Research bank) were maintained in DMEM (Nissui, Tokyo, Japan) supplemented with 15% FBS.

Flow cytometry was performed after 24 h treatment with hCG (Roto Seiyaku, Osaka, Japan) [16,23]. Cisplatin cytotoxicity (Sigma, Saint Louis, MO) was evaluated using the WST-1 assay [24]. Invasion assays were performed using Boyden chambers [16].

Microarray data. We analyzed published gene expression datasets including GSE2487 and GSE1926 (http://www.ncbi.nlm.nih.gov/geo/), E-MIMR-11 (http://www.ebi.ac.uk/arrayexpress/), the ovarian cancer dataset from Swegene DNA Microarray Resource Center (http://www.oncology.gu.se/forskning/opublicerad.data/MAlog2value.KPartheen06) and the NCI60 dataset (http://dtp.nci.nih.gov/docs/cancer/cancer_data.html). Expression values were compared using Mann–Whitney *U* tests.

Immunohistochemistry, cytological analysis. Immunohistochemical and cytological analysis was performed using the TRO monoclonal antibody (clone 3–11, mouse IgM, 1:100 dilution) [18] as previously reported [16].

RT-PCR. RT-PCR analysis was performed as previously reported [16]. PCR conditions were: 94 °C for 1 min, 51 °C (*TRO*) or 55 °C (*ACTB*) for 1 min, then 72 °C for 2 min for 30 (*TRO*) or 25 (*ACTB*) cycles. Primers included: *TRO*, forward 5′-ACGATGAATGTGGCG ATACT-3′, reverse 5′-AGTGCATTCAAGGCTGTTGG-3′; *ACTB*, forward 5′-CCGCAAAG ACCTGTACGCCA-3′, reverse 5′-TGGACTTGGGAGAGGACTGG-3′.

TRO knockdown. *TRO* suppression was achieved using retroviral [16] infection with shRNAs (pSM2-TRO; RHS1764-9693529 or pSM2c; RHS1763, OpenBiosystems, Huntsville, AL) or lipofection with siRNA duplex (Stealth™ RNA, Invitrogen).

Tumorigenicity assays. Animal care and experimental protocols followed guidelines of the Animal Research Committee, Graduate School of Medicine, Kyoto University. 5×10^6 cells (knockdown or control) were injected IP into 4–6 week old female BALB/C nude mice (CLEA JAPAN, Tokyo, Japan). Each group was subdivided and injected weekly IP with 100 μl PBS ± cisplatin (2 μg/g body weight). Survival time represents the time required to reach endpoints as previously described [24]. Survival curves were analyzed using a Logrank test.

Results and discussion

Genome-wide transcriptional alteration by RAS

RAS-signaling pathway activation is common in ovarian cancer [7,8]; nevertheless its role in tumorigenesis and tumor progression is unclear. We performed a genome-wide gene expression analysis to gain insight into signaling components involved in RAS-induced *in vitro* transformation. Transcriptome analysis of immortalized OSE with or without RAS transformation revealed substantial changes in the gene expression profile. To determine the overall effect induced by RAS, we compared our data to previously published independent datasets (GSE2487 and E-MIMR-11), which were obtained after RAS introduction into immortalized cells, using Pearson correlation of fold change in expression. Among 3552 overlapping genes, expression alterations induced by RAS showed positive correlation with GSE2487 ($r = 0.19$, $P = 7.6 \times 10^{-30}$) and E-MIMR-11 ($r = 0.20$, $P = 1.9 \times 10^{-34}$). Our data showed 215 genes were upregulated (Supplementary Table 1), among which the KEGG pathway "MAPK signaling" was enriched ($P < 0.01$) using GATHER [25], while 355 genes were downregulated by RAS (Table 1). Such downregulation may overcome barriers to tumor progression mediated by RAS. Among these, we further analyzed

Table 1
cDNA microarray analysis between immortalized OSE cells (IOSE) and Ras-transformed IOSE cells (ras-IOSE) showing TRO downregulation through *in vitro* transformation by Ras

UniGene ID	Locus link ID	Gene symbol	Gene name	Fold change ($\log_2 X$)
Hs.458414	8519	IFITM1	Interferon-induced transmembrane protein 1 (9–27)	−4.44
Hs.270978	4232	MEST	Mesoderm-specific transcript homolog (mouse)	−4.00
Hs.524224	715	C1R	Complement component 1, *r* subcomponent	−3.63
Hs.388347	143381	LOC143381	Hypothetical protein LOC143381	−3.63
Hs.464419	26270	FBXO6	F-box protein 6	−3.47
Hs.147041	55713	ZNF334	Zinc finger protein 334	−3.45
Hs.518525	2752	GLUL	Glutamate-ammonia ligase (glutamine synthase)	−3.09
Hs.1706	10379	ISGF3G	Interferon-stimulated transcription factor 3, gamma	−3.01
Hs.434971	7216	TRO	Trophinin	−2.92
Hs.166120	3665	IRF7	Interferon regulatory factor 7	−2.92
Hs.3447	26022	DKFZP564K1964	DKFZP564K1964 protein	−2.90
Hs.529317	55008	HERC6	Hect domain and RLD 6	−2.89
Hs.7337	55266	TMEM19	Transmembrane protein 19	−2.87
Hs.513439	2581	GALC	Galactosylceramidase (Krabbe disease)	−2.82
Hs.83077	3606	IL18	Interleukin 18 (interferon-gamma-inducing factor)	−2.81
Hs.170473	26499	PLEK2	Pleckstrin 2	−2.77

The table lists genes for which the $\log_2$ scaled expression fold change of ras-IOSE/ IOSE is <-2.5.

TRO since it was reproducibly downregulated in the RAS-transformed cells, several reports describe the role of TRO or the related MAGE-D1 in tumor phenotypes [19–21] and because an anti-TRO antibody was available [18].

Impact of TRO expression in cancer cells on cisplatin sensitivity and invasiveness

Among eight ovarian cancer cell lines analyzed, OVCAR-3, CH1, TYK-nu, and A2780-s are positive for *TRO* mRNA (Fig. 1A). *TRO*-negative cell lines included two cisplatin-resistant derivatives (TYK-nu CP-r, and A2780-cp), suggesting a possible relationship between TRO expression and cisplatin sensitivity. We therefore measured the drug concentration that inhibits 50% of cell growth (IC_{50}) in these cell lines. Cisplatin IC_{50}s for TRO(+) cell lines were significantly lower than those for TRO(−) ones (Fig. 1B, $P < 0.05$). To further confirm this relationship, we analyzed two-independent expression microarray datasets. The NCI60 database consists of gene

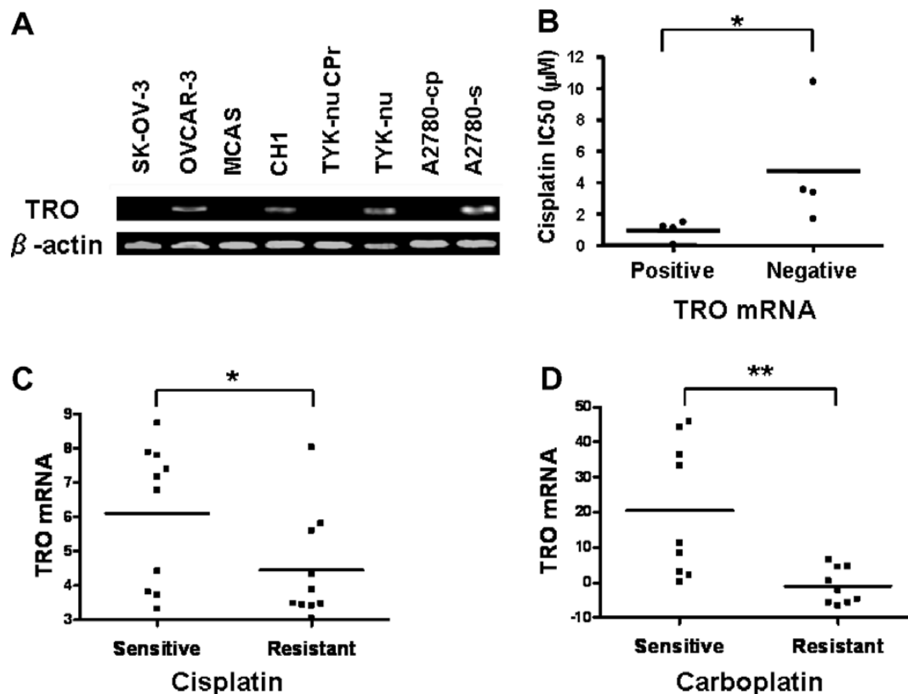

Fig. 1. Expression of TRO in cancer cell lines. (A) *TRO* mRNA for ovarian cancer cell lines was analyzed by RT-PCR. β-Actin served as an internal control. (B) Cisplatin IC_{50} values for cell lines positive for *TRO* mRNAs were lower than those negative for *TRO* mRNAs $^{*}P < 0.05$. (C) Comparison of *TRO* mRNA expression between the ten most cisplatin sensitive and resistant cell lines in the NCI60 database. $^{*}P < 0.05$. TGI50 values for cisplatin were negatively correlated with *TRO* mRNA expression ($P = 0.079$, not shown). (D) Comparison of *TRO* mRNA expression between primary cultured ovarian cancer cells sensitive and resistant to carboplatin. $^{**}P < 0.01$.

expression microarray data for 59 cancer cell lines alongside chemosensitivity profiles for thousands of drugs [26]. We compared *TRO* mRNA levels between the ten most cisplatin sensitive and resistant NCI60 cell lines. Cisplatin-sensitive cells expressed higher levels of *TRO* than resistant cells (Fig. 1C, $P < 0.05$). Similarly, carboplatin-sensitive cancer cells showed higher *TRO* mRNA expression than resistant cancers in GSE1926, in which primary cultures from ovarian carcinomas were characterized as carboplatin sensitive or resistant [27] (Fig. 1D, $P < 0.05$). These findings support our observations and strongly implicate an important correlation between the level of *TRO* expression and platinum sensitivity.

We next examined the functional consequence of *TRO* suppression by RNAi. We introduced *TRO* shRNAs into A2780-s cells. RT-PCR analysis confirmed effective *TRO* suppression (Fig. 2A). The cisplatin IC_{50} for A2780-s shTRO cells was higher than control cells (8.55 ± 1.35 μM versus 2.47 ± 0.56 μM; Fig. 2B, $P < 0.01$), demonstrating that modulation of *TRO* expression *in vitro* alters cisplatin sensitivity.

To assess the potential *in vivo* modulatory effect on cisplatin sensitivity, we inoculated these cells into the peritoneal cavity of nude mice and simultaneously administered cisplatin. All mice formed solid tumors in their peritoneal cavity, and the mean survival of the A2780-s sh control group was extended from 43 ± 2.8 to 71 ± 8.2 days by cisplatin treatment (Fig. 2C, $P < 0.05$), while the A2780-s shTRO group was not significantly changed (34.0 ± 6.0 versus 31.5 ± 3.5 days; $P = 0.176$).

Platinum drugs elicit DNA damage in cancer cells resulting in apoptosis. Forced expression of anti-apoptotic proteins like BCL2 and IAPs decrease cisplatin sensitivity *in vitro*, and elevated expression correlates with cisplatin resistance of tumors in clinical studies [28]. MAGE-D1 induces apoptosis by degrading XIAP [29]. Moreover, deregulation of RAS pathways confers cisplatin resistance in ovarian cancers [28,30], and RAS activity is indeed higher in A2780-cp cells than A2780-s cells [31]. These observations suggest that TRO may modulate platinum sensitivity through a pro-apoptotic mechanism and via an effect on RAS signaling.

We performed several *in vitro* assays to assess other potential phenotypic changes resulting from siRNA-mediated *TRO* modulation. Near complete suppression of *TRO* expression was achieved in siTRO-2 transfected cells (Fig. 2A). There were no significant differences in proliferation- or anchorage-independent growth capacity between control and siTRO-2 transfected cells (not shown). We tested the effect of *TRO* modulation on invasiveness using OVCAR-3 cells, which are larger than A2780-s cells whose extremely small size precluded testing (Supplementary Fig. 1). The invasive potential of OVCAR-3 was significantly increased by *TRO* suppression (Supplementary Fig. 2, $P < 0.01$) indicating that TRO contributes to phenotypic heterogeneity by influencing ovarian cancer cell invasiveness and cisplatin sensitivity.

The epithelial–mesenchymal transition (EMT) is an important step in morphogenesis and in the progression of the primary tumor toward an invasive phenotype and involves epithelial cells acquiring fibroblastoid morphology accompanied by loss of differentiation with increased motility [14,32]. TRO facilitates embryo implantation, an example of EMT, through homophilic adhesion between blastocyst and maternal cells [18]. MAGE-D1 disrupts actin cytoskeleton organization and lamellipodia formation when overexpressed [33], processes that characterize EMT. EMT also accompanies RAS-mediated transformation, augmenting anchorage-independent growth and migration [34]. For example, elevated H-RAS induces

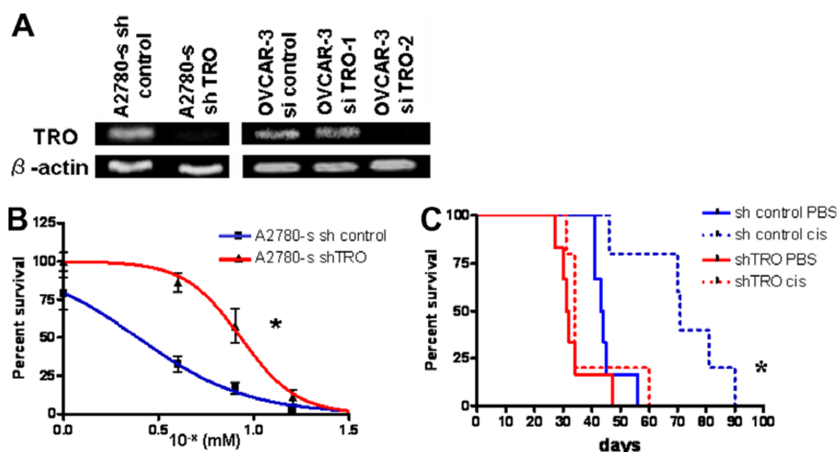

Fig. 2. Effects of TRO suppression. Control shRNAs or TRO shRNAs were introduced into A2780-s. OVCAR-3 cells were transfected with Stealth™ RNA (control, si TRO-1, or si TRO-2). $^{*}P < 0.01$. (A) Expression of *TRO* mRNA in the cells following introduction of shRNA or siRNA transfection. (B) Chemosensitivity assay of A2780-s sh control cells and A2780-s shTRO cells; the IC_{50} value of A2780-s shTRO cells (▲, red line) was significantly higher (8.551 μM) than that of A2780-s sh control cells (■, blue line; 2.468 μM) ($P < 0.01$). (C) Mouse xenograft experiments. Nude mice inoculated with A2780-s sh control cells ($N = 6$) or A2780-s sh TRO cells ($N = 5$) were treated with PBS or cisplatin. Red, survival curves of mice inoculated with A2780-s sh TRO cells. Blue, survival curves of mice inoculated with A2780-s sh control cells (solid line, PBS treated; dotted line, cisplatin treated). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nuclear accumulation of Smad2, both of which are essential for the EMT [32]. Twist, a transcription factor induced by RAS, contributes to metastasis by promoting the EMT [35]. Based on our results, TRO is also involved as a potential effector of RAS in the process of EMT in ovarian cancer cells. By extension, TRO may affect prognoses of ovarian cancer patients.

TRO is a prognostic factor in ovarian cancer

We used immunohistochemistry to examine TRO expression in primary ovarian cancers. Among 56 cases of epithelial ovarian cancer, 29 cases were positive for TRO (Fig. 3A, Supplementary Table 2). Kaplan–Meier analysis for 42 stage III–IV cases revealed that the mean progression-free survival of the 23 TRO positive cases was 23.2 months, versus 10.7 months for TRO negative cases (Fig. 3B, $P < 0.05$). There was no significant difference between these groups in age, tumor histology, tumor debulking status or chemotherapeutic regimen. We further assessed *TRO* expression in ovarian cancers using the Swegene expression microarray dataset. Among 54 stage III serous adenocarcinomas, *TRO* expression was significantly higher in the patients that achieved 5-year survival versus those that did not (Fig. 3C, $P < 0.05$). These observations demonstrate that *TRO* is downregulated in tumors with worse outcome and suggest its preventive role in ovarian tumor progression.

During blastocyst implantation, *TRO* expression is induced around the implantation site by human chorionic gonadotropin (hCG) [18,23]. It is presently unclear whether there is a similar relationship between hCG and *TRO* expression in cancer. We therefore examined the effect of hCG on *TRO* expression in ovarian cancer. Flow cytometry analysis indeed detected that *TRO* expression in OVCAR-3 cells with luteinizing hormone/hCG receptor (LHCGR) [36] increased in a dose-dependent manner with hCG addition, while that in SK-OV-3 cells without LHCGR [36] was unchanged (Supplementary Fig. 3). This suggests that *TRO* expression is regulated by hCG as well as by RAS. While hCG activates the Ras-Erk1/2 pathway in mouse Leidig tumor cells [37], the interaction between hCG and RAS signaling is unclear in ovarian cancer, in which aberrant RAS activation is dominant enough to overcome the hCG effects on the RAS pathway.

To further understand the relationship between TRO and hCG/LHCGR, we analyzed *TRO* expression among 27 ovarian cancers for which *LHCGR* expression had previously been reported [36]. 72.2% of the LHCGR positive cases were also positive for TRO, significantly higher than the LHCGR negative cases (22.2%, $P < 0.05$, Fisher's exact test; Supplementary Table 3). Thus, *TRO* and *LHCGR* expression significantly overlap in ovarian cancers. LHCGR-positive ovarian cancers have a better prognosis than those negative [36], presumably because TRO may play a role in LHCGR positive cancers by modulating cisplatin sensitivity.

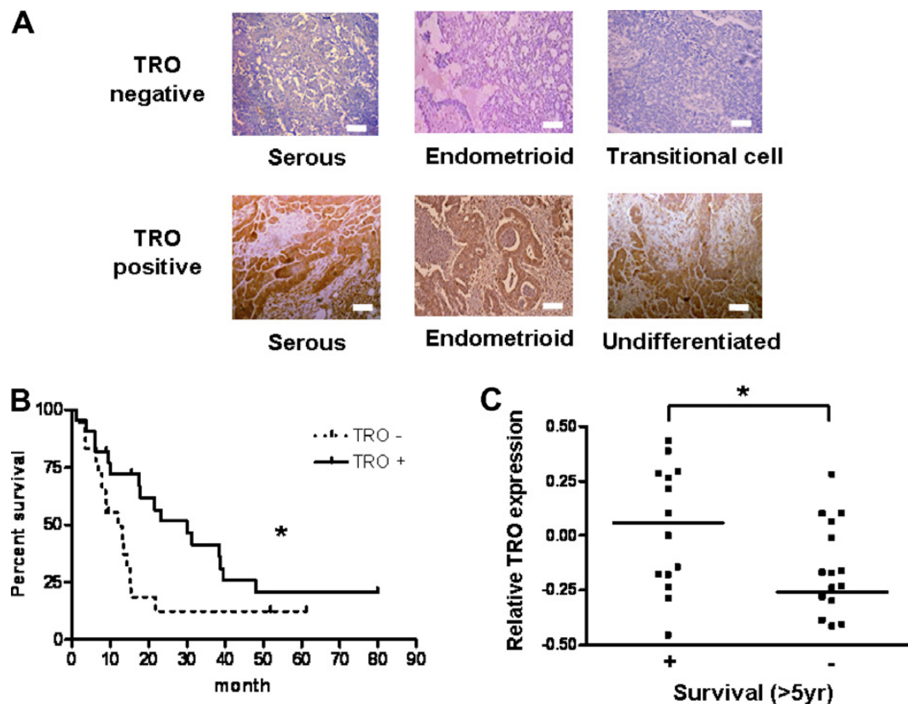

Fig. 3. TRO expression in ovarian cancer. (A) Immunohistochemical detection of TRO expression in ovarian cancer. Top row, ovarian cancers negative for *TRO* (serous, endometrioid, and transitional cell). Bottom row, ovarian cancers positive for *TRO* (serous, endometrioid, and undifferentiated). Bar, 100 μm. (B) Kaplan–Meier analysis for progression free survival (PFS) of stage III–IV ovarian cancer cases. $^{*}P < 0.05$. (C) Validation study of *TRO* mRNA expression in ovarian cancer. $^{*}P < 0.05$.

In conclusion, *TRO* expression is suppressed by RAS through *in vitro* transformation of OSE cells. TRO modulates platinum sensitivity and invasiveness of ovarian cancer cells, and its expression is associated with better prognosis of patients with advanced ovarian cancers. These observations point to TRO as a potent marker for diagnostic and therapeutic intervention.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.070.

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