TCTP Silencing in Ovarian Cancer Cells Results in Actin Cytoskeleton Remodeling and Motility Increase

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Abstract: Translationally Controlled Tumor-associated Protein (TCTP) plays a role in a plethora of normal and cancer cell functions including cell cycle progression, cell growth and metastasis. Our previous studies showed that TCTP interacts with cellular cytoskeleton and is localized, in cell-type specific manner, on actin filaments in various types of ovarian cancer cells. Here we used small interfering RNA (siRNA) for silencing TCTP expression in human ovarian surface epithelial noncancerous cell line HIO180, ovarian carcinoma cell lines SKOV3 and OVCAR3 and analyzed effect of TCTP silencing on actin cytoskeleton and cell motility. We show that a down regulation of TCTP caused dramatic restructuring and redistribution of actin filaments in HIO180, SKOV3 and OVCAR3 cells and resulted in cell motility increase. This previously unidentified dependence of actin cytoskeleton remodeling and cell motility on TCTP level might be responsible for high metastatic potential and aggressiveness of ovarian cancer cells and will help to pinpoint novel targets for anticancer therapies.

Keywords: TCTP, siRNA, ovarian cancer cells, actin cytoskeleton, cell motility.

INTRODUCTION

The translationally controlled tumor protein (TCTP) is conserved in all eukaryotic cells. It plays a role in the regulation of multitude of functions such as cell death (apoptosis), cell growth, cell division and cell cycle, and as such it is extremely important for cancer formation, progression and metastasis [1-6]. Several studies showed that highly metastatic and poorly differentiated tumors express very high level of TCTP [7, 8] and that a down regulation of TCTP expression in cancer cells induces apoptosis and reverts malignant phenotype [9-11]. Numerous studies of various cancer types, including human ovarian epithelial cancer, have determined that oncogenic transformation and metastasis result from the profound changes in the distribution and organization of cellular cytoskeleton [12-28]. It has been also shown that the changes in cytoskeleton architecture are responsible for the high migratory activity of ovarian cancer cells [13, 21-26, 28]. Recent studies on different cell types showing localization of TCTP on the cytoskeleton suggest that the role of TCTP in the regulation of cell cycle and cell migration might be related to its cytoskeleton binding ability [29-31].

Our previous light and electron microscopy studies on epithelial ovarian HIO180 cells and the epithelial ovarian cancer cell lines SKOV3 and OVCAR3 with different metastatic potential (SKOV3 cells are more metastatic than the OVCAR3 cells), showed that these cells differ profoundly in the organization and distribution of actin cytoskeleton and that TCTP is localized on actin filaments [32]. In present study we silenced TCTP expression in HIO180, OVCAR3 and SKOV3 cells using small interfering RNA and showed that a down regulation of TCTP caused cell-specific changes in actin cytoskeleton architecture and cell motility.

MATERIALS AND METHODS

Cell Lines

The human ovarian surface epithelial cell line HIO180 and two ovarian carcinoma cell lines, OVCAR3 and SKOV3, were cultured as described in Kloc *et al*. [32].

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RNA Interference

TCTP siRNA interference was performed using OriGen Trilencer-27 siRNA kit and siTran transfection reagent (Origene, Rockville, MD, USA) according to the manufacturer's protocol. Trilencer-27 Universal Scrambled siRNA was used as a negative control. We used three different TCTP siRNA duplexes:

SR304920A-rCrArCrCrArGrGrArCrUrUrArArGrArCrAr ArArUrGrGrGAC, SR304920B – rGrGrArUrCrUrArUr CrArCrCrUrGrUrCrArUrCrArUrArACT and SR304920C – rGrCrArGrArArCrArArArUrCrArArGrCrArCrArUrCr CrUTG, which were further in the text described as siRNA-A, siRNA-B and siRNA-C respectively. These three siRNAs duplexes were from TCTP silencing kit purchased from OriGen Trilencer-27 siRNA kit (cat # SR304920; Origene, Rockville, MD, USA). These duplexes were designed to be effective against all transcriptional variants at human TCTP gene locus. Cells were harvested 24-48 hr post-transfection.

Western Blotting and Antibodies

Cells were homogenized on ice in RIPA buffer (0.15 M NaCl, 1% deoxycholate sodium salt, 1% Triton X-100, 0.1% SDS and 0.01 M Tris-HCl (pH 7.2)) in the presence of a complete protease inhibitor (Roche, Indianapolis, IN). The protein concentration was determined using Bio-Rad protein assay reagents. Following antibodies were used: anti-TCTP and anti-GAPDH rabbit polyclonal antibodies (Abcam, Cambridge, MA, USA). Proteins were separated using SDS-PAGE and blotted with 1:500-1:1000 dilutions of primary antibodies. After intensive washing, blots were incubated in a 1:2000 dilution of HRP-conjugated secondary antibody and developed using the Lumi-Light western blotting substrate (Roche, Indianapolis, IN).

Actin Staining

Cells grown on chamber slides were fixed in 4% formaldehyde (EM grade) in PBS with 0.1% Triton X-100 for 30 min at room temperature. After two 15 min washes in PBS-0.05% Tween 20, the slides were blocked for 2 hr using casein blocking buffer (Bio-Rad) with 0.05% Tween 20 in PBS and than stained overnight with rhodamine-phalloidin (5 µl of methanolic stock solution of 200 U/ml per 200 µl of PBS + 1% BSA) (Molecular Probes, Eugene, OR). After washing in PBS -Tween in the dark, the slides were mounted using an antifade reagent containing Hoechst (nuclear staining) and imaged using a Nikon fluorescence microscope.

Migration Assays

The migration of control and TCTP siRNAs treated (for 24 hr) cells was tested using transmigration assay. In the Millipore QCMT colorimetric cell transmigration assay (Millipore, Billerica, MA, USA) the cell suspensions containing 10^6 cells were loaded in serum free medium into the chambers with 8µm pore size polycarbonate membranes. Cells that migrated through the membrane toward 10% FCS-containing medium were incubated with cell stain solution, extracted and colometric measurement of optical density was performed at 560nm using standard microplate reader.

RESULTS

TCTP siRNA Interference down Regulates TCTP Protein Expression

We transfected three different TCTP specific siRNAs (siRNA A, B, and C) and control universal scrambled siRNA into noncancerous human ovarian epithelial cells HIO180 and epithelial ovarian cancer cells OVCAR3 and SKOV3 and after 48hr isolated total proteins and performed Western blot analysis of TCTP and GAPDH (loading control marker) protein expression. We found that consistently all three siRNAs were effective (although to a different degree) in targeted down regulation of TCTP protein expression in all studied cell lines (Figure **1**).

TCTP Silencing Correlates with Actin Cytoskeleton Remodeling

Actin staining with rhodamine-phalloidin of TCTP siRNA treated cells showed a dramatic changes in the organization of actin cytoskeleton in all studied cell lines (Figures **2-4**). The control (untreated and universal scrambled siRNA treated) HIO180 non-transformed ovarian epithelial cells showed the abundance of thin actin filaments within the whole cytoplasm (Figure **2**). Treatment with any of three TCTP siRNAs caused redistribution of long actin filaments toward the cell periphery and shortening of the filament crisscrossing the cytoplasm and also resulted in a strong accumulation of actin in the nuclei of some cells (Figure **2C**; panel C and C1 show cell treated with siRNA-A). The control OVCAR3 ovarian carcinoma cells showed thick actin filament stress fibers located at the cell periphery (Figure **3**). In OVCAR3 cells treated with TCTP siRNAs we observed

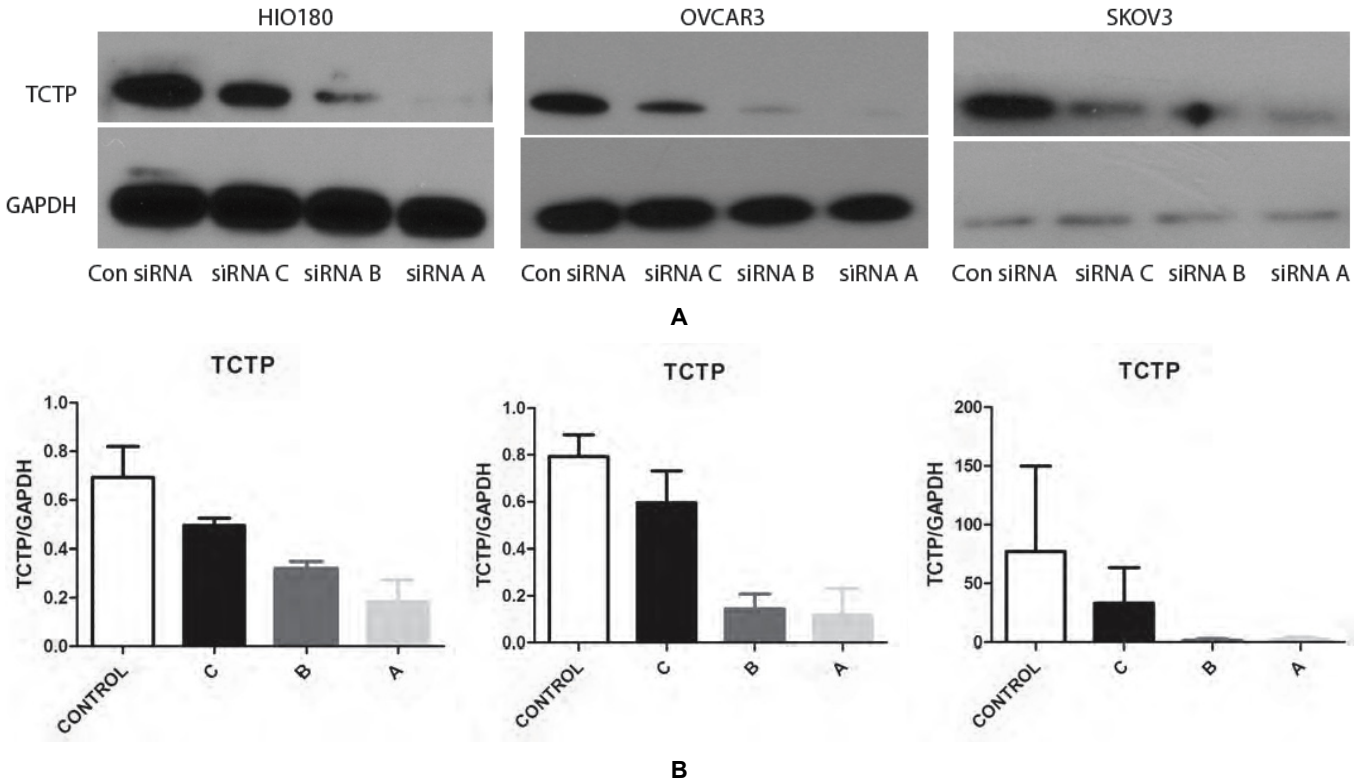


Figure 1: TCTP protein expression after TCTP siRNA silencing.

A, **B**). Noncancerous ovarian epithelial cells HIO180 and two ovarian epithelial cancer cell lines SKOV3 and OVCAR3 were transfected with control scrambled siRNA and three (**A**, **B**, **C**) TCTP-specific siRNAs. At 24-48 hr post transfection cells were processed for Western blot analysis with anti-TCTP antibody (GAPDH expression was used as a loading control marker) and intensity of scanned bands from 3 independent experiments was plotted (as TCTP/GAPDH ratio) in the graphs. The graphs show that all three TCTP siRNAs were effective (although to a different degree) in a down regulation of TCTP protein expression in all studied cell lines.

two distinct phenotypes of actin filaments distribution. Cells treated with siRNA-B or siRNA-C showed long actin filaments present within the whole cytoplasm (Figure **3C**, C1). However, in cells treated with siRNA-A the long actin filament were very sparse (Figure **3D**, D1), and majority of cell cytoplasm was filled with short actin filaments or comets (Figure **3D**, D1), which resembled actin phenotype observed in control SKOV3 cells (Figure **4A** and **B**). The SKOV3 cells treated with any of three TCTP siRNAs showed the abundance of very well defined long actin filaments crisscrossing the cytoplasm (Figure **4**; image in panel C, C1 shows cell treated with siRNA-B).

TCTP Silencing and Actin Cytoskeleton Remodeling Increase Cell Motility

In order to assess if the TCTP silencing and corresponding changes in actin cytoskeleton influence cell motility we performed migration assays on control and TCTP siRNAs treated cell lines. This assay showed that TCTP down regulation (with any of three different TCTP siRNAs) increased by about 56% the motility of HIO180 cells (Figure **5**). The motility of SKOV3 cells increased about 30% after treatment with TCTP siRNA-A, and about 17% and 15% after treatment with siRNA-B or siRNA-C (Figure **5**). Interestingly, the motility of OVCAR3 cells decreased by about 20% after treatment with TCTP siRNA-A, and increased by 20% after treatment with TCTP siRNA-B or siRNA-C (Figure **5**).

DISCUSSION

Our study established that TCTP silencing resulted in profound changes in the organization of actin cytoskeleton in noncancerous ovarian epithelial HIO180 cells and ovarian cancer cells SKOV3 and OVCAR3. The changes in the architecture of actin cytoskeleton correlated with the increased migratory activity of these cells. Because the increase in migratory activity is known to positively correlate with cancer cell metastatic potential these results suggest that the TCTP may be involved in regulation of cancer cell metastasis. We found that TCTP silencing in

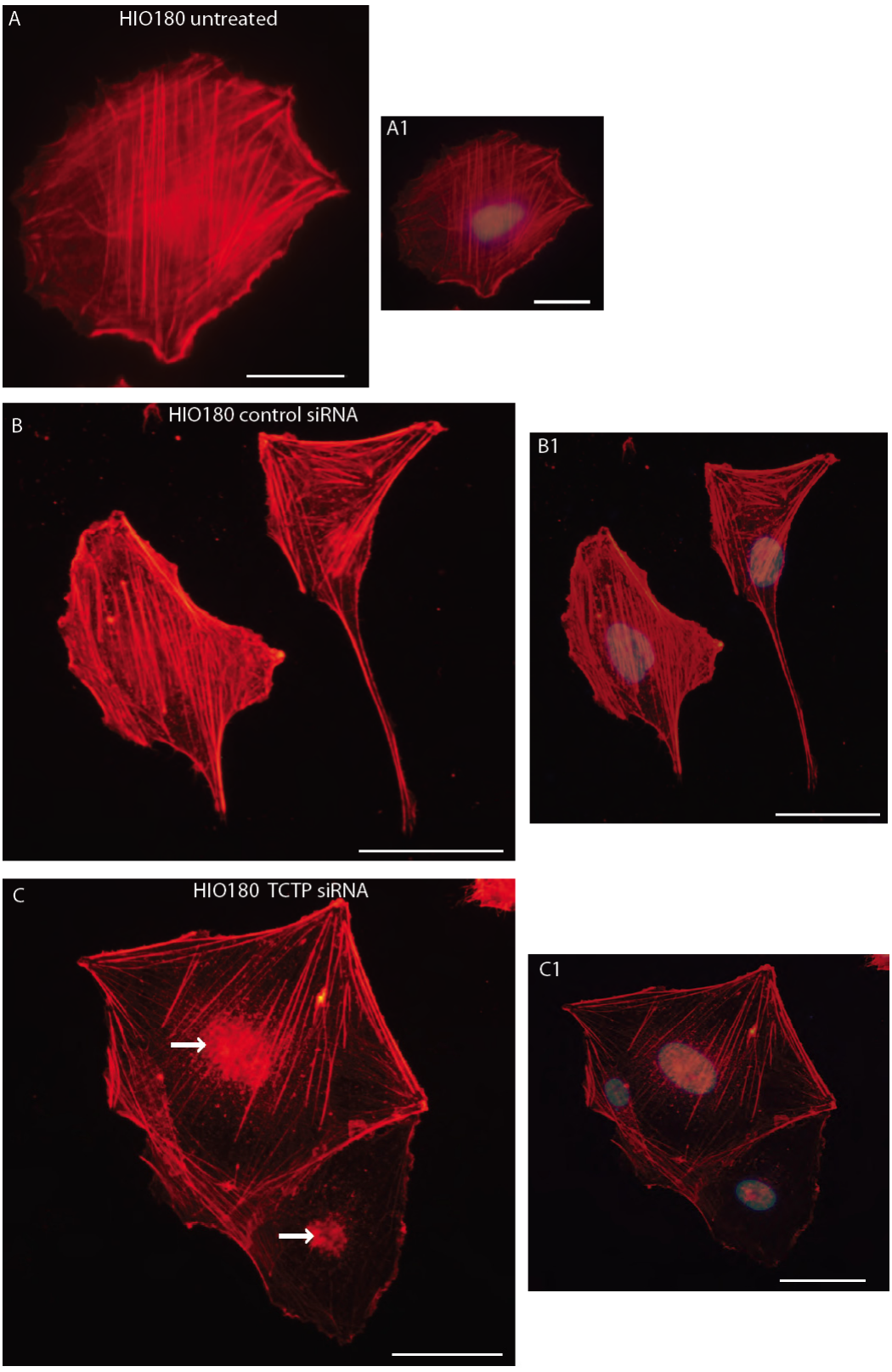


Figure 2: Actin distribution in the HIO180 ovarian surface epithelial cell line after TCTP silencing.

Control (untreated and scrambled siRNA treated) and TCTP siRNA-treated HIO180 cells were grown on chamber slides, fixed, stained with rhodamine-phalloidin and counterstained with Hoechst. **A**, **B**) Control cells show long actin filaments crisscrossing cell cytoplasm and accumulation of actin in cell nuclei (arrow). **C**) TCTP siRNA (siRNA-A, -B or –C) treatment resulted in shortening of actin filaments within the cytoplasm and redistribution of long actin filaments toward the cell periphery. Image shows cell treated with siRNA-A. Nuclei show very strong accumulation of nuclear actin (arrow). **A1-C1**) Merged images of actin staining with Hoechst stained nuclei (blue). The bar is equal to 50 μm.

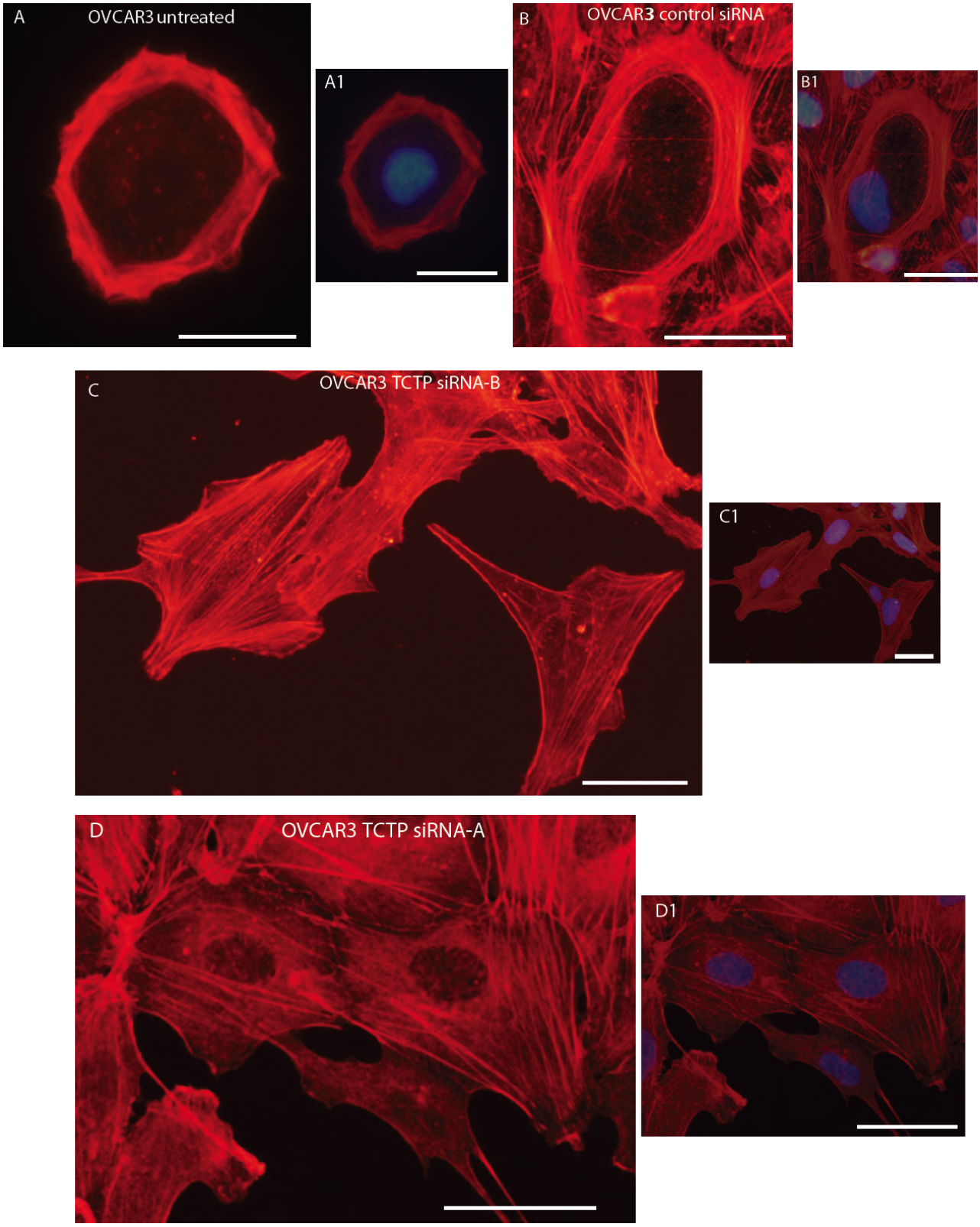


Figure 3: Actin distribution in the OVCAR3 ovarian epithelial cancer cell line after TCTP silencing.

Control (untreated and scrambled siRNA treated) and TCTP siRNA treated OVCAR3 cells were grown on chamber slides, fixed, stained with rhodamine-phalloidin and counterstained with Hoechst. **A**, **B**) Control cells show actin filaments concentrated at the cell perihery. **C**) Treatment with TCTP siRNA-B or siRNAC (not shown) resulted in abundant formation of long actin filaments within the cell cytoplasm. **D**) In cells treated with TCTP siRNA-A cytoplasm is filled with short actin filaments or comets and very few long actin filaments. **A1-D1**) Merged images of actin staining with Hoechst stained nuclei (blue). The bar is equal to 50 μm.

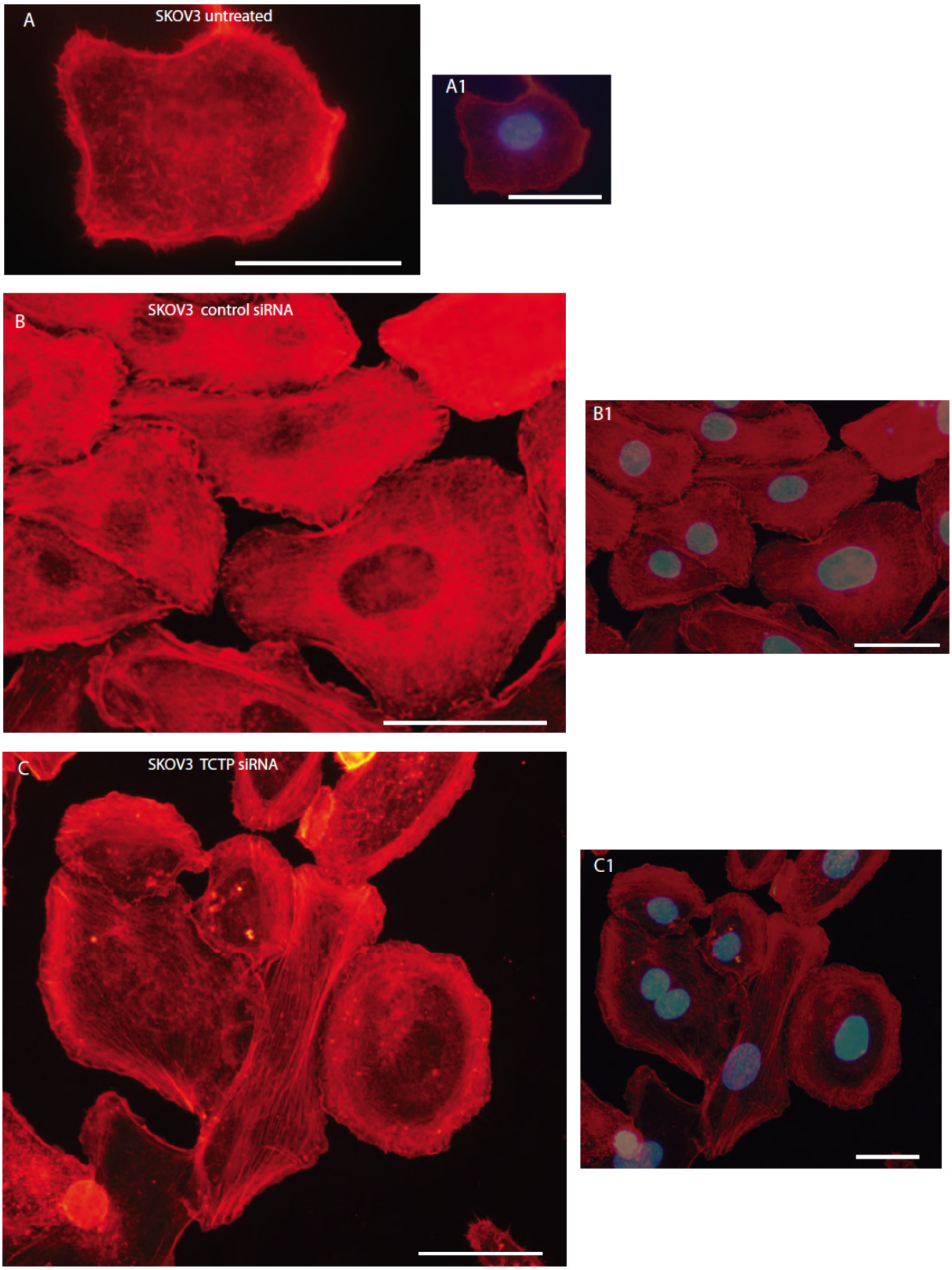
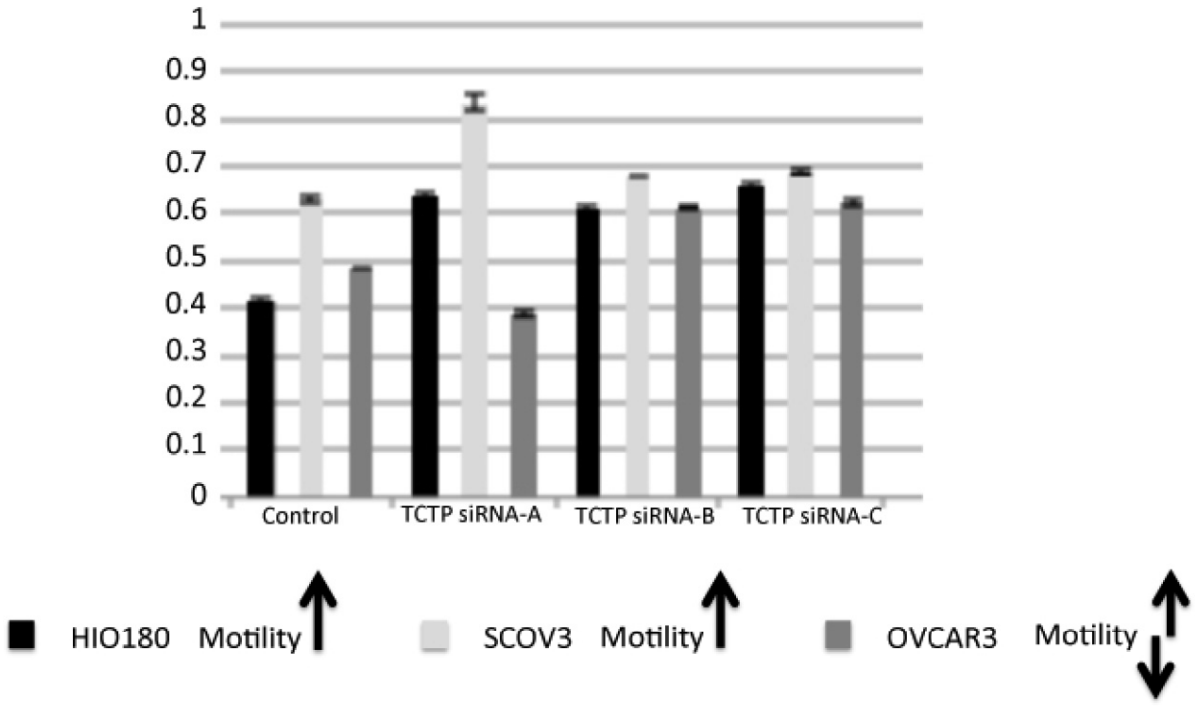


Figure 4: Actin distribution in the SKOV3 ovarian epithelial cancer cell line after TCTP silencing.

Control (untreated and scrambled siRNA treated) and TCTP siRNA treated SCOV3 cells were grown on chamber slides, fixed, stained with rhodamine-phalloidin and counterstained with Hoechst. **A**, **B**) Control cells show cell cytoplasm filled with short actin filaments or comets. **C**) Treatment with any of three TCTP siRNAs resulted in the redistribution of actin toward cell periphery and formation of long actin filaments crisscrossing the cytoplasm. Image shows cell treated with siRNA-B. **A1-C1**) Merged images of actin staining with Hoechst stained nuclei (blue). The bar is equal to 50 μm.



Cell type	Control	siRNA-A	siRNA-B	siRNA-C
HIO180	100%	157%	150%	162%
SKOV3	100%	133%	117%	115%
OVCAR3	100%	79%	122%	124%

Figure 5: TCTP silencing increases migratory activity of ovarian epithelial normal and cancer cells.

Graph (**A**) and percentage changes (**B**) representation of migratory activity of ovarian noncancerous epithelial HIO180 cells and ovarian epithelial cancer cells SKOV3 and OVCAR3 after TCTP silencing. Y-axis represents values of colometric measurement of optical density performed at 560nm. Bars show average from 3 experiments. Standard deviation was calculated in Excel.

HIO180 cells correlated with the redistribution of long actin filaments toward the cell periphery and shortening of the filaments crisscrossing the cytoplasm and increase in cell motility. Interestingly, the silencing of TCTP in OVCAR3 cells resulted, depending on the siRNA used (siRNA-A versus siRNA-B or siRNA-C), in two distinct actin organization and motility phenotypes. It is possible that the replacement of peripheral actin stress fibers with short comet-like filaments caused cell motility increase ("fast" phenotype) and that the replacement of peripherally located actin stress fibers with the long actin filaments crisscrossing the cytoplasm caused cell motility decrease ("slow" phenotype) or vice versa. Although at present we do not have any indication if or which of these two scenarios are true the studies of progressive ovarian cancer cells MOSE in mouse model showed that during the malignant progression of MOSE- E (early) to MOSE-L (late) cells, the long actin stress fibers are replaced by very short actin filaments [14] and that MOSE-E cells are more rigid than MOSE-L cells [17]. These authors suggest that the changes in actin organization are responsible for the increased cell deformability, which in turn correlates with the progression from a noncancerous to a malignant phenotype [14, 17]. The correlation between actin cytoskeleton changes and cell motility has been also described in colorectal cancer and breast cancer cells [33, 34]. Our study clearly shows that the changes in the organization of actin cytoskeleton correlated with the changes in cell motility in all studied here cell types. These results agree with numerous findings that in various cancer cells the changes in the organization of the cytoskeleton are responsible for oncogenic transformation and increased cell motility and invasiveness [19-26, 35, 36]. Similarly, the changes in the expression of cytoskeletal genes and proteins during the progression from low- to high-grade ovarian cancer were also reported for several human ovarian cancer cell lines (i.e., ES-2a, TOV-21Gb, RMG-Ic, OVMANAd, OVISEd, OVASd, OVTOKOd, OVSAYOd, KKd, SMOV-2d, TOV-81D and TOC-112D), [37, 38].

The cancer formation, progression and metastasis consist of a very complex processes involved in transformation of cell fate, growth of primary tumor, cell

motility and vascular invasion, and TCTP as a multifunctional molecule may potentially participate in or regulate some or all of these events. Recent studies on androgen-dependent prostate cancer LNCaP cells [39] and human colon cancer LoVo cells transfected with the phosphatase of regenerating liver-3 (PRL-3) gene (LoVo-PRL-3 cells [40] showed that siRNA mediated silencing of TCTP changed cell proliferation [39], migration, invasion and viability [40]. Our present study is the first report showing the existence of very strong correlation between the level of TCTP and organization of actin cytoskeleton and cell motility. We previously showed that TCTP is localized on actin filaments [32], which strongly suggests that TCTP may be directly involved in actin remodeling and cell motility. Although, as far as we know, there is no information available on the role of TCTP in actin remodeling this is very important in the context of recent studies indicating that actin remodeling can be used as a biomarker for chemoprevention trials [45]. These authors tested the effect of epidermal growth factor receptor tyrosine kinase inhibitor (erlotinib) on actin remodeling in urothelial cells (bladder cancer model system) *in vitro* and concluded that actin remodeling may be related to epithelial to mesenchymal transition and increased cell motility and metastasis.

Another interesting finding in our study is that the silencing of TCTP in HIO180 cells resulted in high accumulation of actin in cell nuclei. Although the presence of actin in cell nuclei was already reported in 1969 its nuclear function has been debated and remained a mystery for nearly four decades [41]. In the last decade numerous studies showed that nuclear actin is essential for chromatin remodeling and RNA transcription, intra-nuclear transport and export of RNAs and proteins and also for nuclear envelope assembly [41-44]. Recent studies of Miyamoto *et al*. [44] showed that nuclear actin is involved (in polymerization-dependent manner) in transcriptional reprogramming through its binding to regulatory regions of the genes. These authors showed that nuclear actin polymerization is regulated by an actin signaling protein Toca-1 and plays a role in the transcriptional reactivation of the pluripotency gene Oct4 [44]. We believe that the correlation between the level of TCTP expression and the nuclear actin abundance, which we observed in our present study, may indicate an existence of a novel and underappreciated function of TCTP signaling in regulation of gene expression through its involvement in the nuclear actin polymerization pathway.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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