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Effect of tunicamycin and calpeptin on cell migration and signaling molecules in fibronectin adherent ovarian cancer cell

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This study aims to investigate the effect of Tunicamycin (Tu), which increases intracellular calcium, and calpeptin (Cp), an inhibitor of calpain 1 and 2 enzymes, on cell migration and molecules involved in migration in fibronectin (FN) adherent normal and ovarian cancer cells. The effect of Tu, Cp and, Tu and Cp together (Tu-Cp) treatments on FN-adherent/non-adherent IHOSE-SV40 normal and SKOV-3 ovarian cancer cells on cell migration was tested with real time cell analysis method. Protein expressions of p-FAK, Pyk2, p-Src, Cdc42 and Rac1 signal molecules were determined by western-blot method. Localizations of cytoskeleton proteins of p-FAK, p-paxillin and vinculin were examined by immunofluorescence method. The results show that ovarian cancer cells must adhere to FN for migration, while normal ovarian cells can migrate without adhering to FN. FN stimulated protein expressions of p-FAK in IHOSE-SV40 cells and p-Src and Rac1 in SKOV-3 cells. Tu, Cp and Tu-Cp treatments significantly inhibited cell migration in both FN-adherent normal and ovarian cancer cells at 24 hours. In particular, Tu treatment in normal and Cp treatment in cancer cells have a decreasing effect on the expression of signaling molecules. In conclusion, we showed in this study that FN stimulates different signaling molecules involved in migration in normal and ovarian cancer cells. Additionally, Tu, Cp, and Tu-Cp treatments inhibited significantly cell migration in FN bound normal and ovarian cancer cells. Tu inhibited the migration of FN-adherent ovarian cancer cells more effectively than Cp and combined Cp and Tu applications. As a result, this study indicated that Cp might be promising agent for ovarian cancer treatment due to its down regulatory impact on the Rac1/Cdc42 proteins and migration inhibitory effect.

Keywords: Calpain, SKOV-3, IHOSE-SV40, Cdc42

Ovarian cancer ranks eighth in the world in terms of mortality rate among women, with a mortality rate of 4.2% in total population in 2020[1]. In recent years, a noticeable rise in the number of cases has been reported among younger women. Ovarian cancer is often detected in its advanced stages, rendering it the most fatal among gynecological cancers[2]. For these reasons, the research continues to elucidate the molecular mechanism of ovarian cancer to develop new and effective treatment tools and methods against this cancer. In recent studies focusing on ovarian cancer, it was found that *Agrimonolide* showed the ability to suppress the proliferation, migration, and invasion in SKOV-3 cells[3]. Blocking SUGT1 leads to a reduction in proliferation and migration in ovarian cancer[4]. In a study on SKOV-3 cells, the fibroblast growth factor 8 gene was found to affect cell survival, adhesion to the extracellular matrix, and migration[5]. Previous studies have indicated that tripartite motif containing 47 acts as an oncogene in ovarian cancer, and silencing it has been shown to inhibit cell invasion and migration[6].

Cell migration is involved in appropriate immune response, wound repair, tissue homeostasis, and pathologies states such as cancer metastasis[7]. Focal adhesion (FA), essential for cell migration, are protein clusters situated near the plasma membrane. They serve as both mechanical anchors and signal hubs within the cell. A crucial aspect of cell migration is the breakdown or replacement of FAs at the trailing edge of the cell. This process is mediated by Ca^{2+}/calpain-dependent proteolysis and degradation[8]. Ca^{2+}, secondary messenger, plays a role in many cellular processes such as cell growth, migration, signalling, and apoptosis. Ca^{2+} dysregulation is associated with cancer progression and chemoresistance[9]. Calpains, which are calcium-activated neutral cysteine proteinases, play roles in many physiological processes such as cytoskeletal remodeling, apoptosis, cellular proliferation and migration. The primary components of the calpain system consist of calpain 1 (CAPN-1), which is

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triggered at μM Ca^{2+} levels, calpain 2 (CAPN-2), activated at mM Ca^{2+} levels, and their regulator calpastatin. Calpain plays a role in cancer progression by taking part in the proteolysis of their specific substrates, which are signaling molecules during cancer progression[10]. Calpain plays a role in cell migration at FA sites by cleaving FA-associated proteins, such as Focal adhesion kinase (FAK), paxillin, vinculin, and talin. By inhibiting calpain activity, cell detachment at the rear part of the cell, which is essential for cell migration, is compromised, leading to reduced cell migration ability[11]. Tumor cell proliferation, migration and invasion can be suppressed[12]. Calpeptin (Cp), an inhibitor of CAPN-1 and CAPN-2, holds great potential as an antitumor drug for cancer treatment. Research investigating the impact of Cp on pancreatic cancer demonstrated notable reductions in tumor volume and weight, as well as effective suppression of proliferation, migration, and invasion[13].

Tunicamycin (Tu), an inhibitor of N-glycosylation, increases Ca^{2+}release from the endoplasmic reticulum[14]. It inhibits the migration, proliferation and invasion of cancer cells[15]. Recent research focusing on uncovering its molecular mechanism suggests that Tu may be a potential anti-cancer agent for cancer treatment and its molecular mechanism has also been the focus of research. Since fibronectin (FN) protein is known to be effective during the migration of ovarian cells to the abdominal region with ascites *in vivo* in ovarian cancer[16]. We demonstrated the effects of CAPN-1/2 via Cp and intracellular Ca^{2+} flux via Tu on cell migration and signaling molecules involved in migration in FN-adherent normal and ovarian cancer cells. This study will contribute to the understanding of the role of calpains, which is shown as a treatment target in cancer, and Tu, which is shown as an anti-cancer agent, in cell migration.

Materials and Methods

Cell culture

In experimental study, immortalized human ovarian epithelial cells-SV40, IHOSE-SV40 (ABMGOOD T1074), for a normal and SKOV-3 (ATCC® HTB-77™) cell line for ovarian adenocarcinoma cancer were used. IHOSE-SV40 cells were grown in culture medium containing Prigrow I medium (ABMGOOD TM001), 1% penicillin-streptomycin (P4333), and 10% Foetal Bovine Serum (FBS) (HyClone SV3016-03) in T25 flasks at 37°C. SKOV-3 cells were incubated with DMEM (Sigma D6429), 10 % FBS, 1 % penicillin-streptomycin, 0.1 mM MEM Non-essential amino acids (Sigma Aldrich, UK) at 37°C, 5% CO_2 atmosphere.

Quantitative determination of cell migration with xcelligence real-time cell analysis

The effects of 24 μM Tu (Tunicamycin T7765, Sigma, UK), 50 μM Cp (calpeptin, sc-202516) and their combination (Tu-Cp) on cell migration were tested. In order to determine the dose and duration of Cp and Tu used in our study, we followed Zeng *et al.*[17], Zeng *et al.*[18] and Sarı Kılıcaslan and İncesu[19]. In our previously published research, we showed that 50 μM Cp and 24 mMTu effectively inhibited CAPN-1/2 enzyme activity for 12- hour[19].

Cell migration was quantitatively analyzed in an incubator (37°C, 5% Carbone dioxide) environment using the Real Time Cell Analysis (RTCA) system. The study was conducted by utilizing either FN-coatedor FN-uncoatedCIM-plate 16. FN at a concentration of 50 μg/ml was applied to the wells located in the upper chamber of the CIM plate. Subsequently, a cell solution containing 10% FBS as a chemoattractant was introduced into the wells of the lower chamber. IHOSE-SV40 (6×10^4 cells/ml) and SKOV-3 (6×10^4 cells/ml) cells were seeded with serum-free medium into the wells. Two hours after cell seeding, 24 μM Tu (Tunicamycin T7765, Sigma, UK), 50 μM Cp (calpeptin, sc-202516), and both 24 μM Tu and 50 μM Cp (Tu-Cp) together were applied to the cells. The cell migration was recorded for 24- hour by taking a 15-minute measurement with the RTCA-DP device. The results are given as cell index value over time. Each experiment was repeated twice independently of each other (n=4). In the data analysis, as a general rule, a positive cell index value indicates cell migration.

Immunolocalization

Intracellular localizations of p-FAK, p-paxillin and vinculin proteins were demonstrated in IHOSE-SV40 and SKOV-3 cells. IHOSE-SV40 (5×10^4 cells/ml) and SKOV-3 (5×10^4 cells/ml) cells were seeded in FN coated (FN-adherent group) or FNuncoated (FN-non-adherent group)24-well plates. Tu (24 μM), Cp (50 μM) and Tu-Cp were pre-administered to the cells for 1- and 12-hours. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and blocked with 0.1% BSA. Antibodies to p-FAK (sc-374668), p-paxillin (sc-365020), vinculin (sc-73614) were applied to each well for overnight at

+4°C, then the cells were incubated with FITC-bound with secondary antibody (sc-2010) for 1 hour. Lastly, the cells were observed and photographed under an immunofluorescence microscope (Leica DMI 4000B).

Western blotting

Protein expression levels were investigated by western blot method. Normal and cancer cells (5×10^6 cells/ml) were seeded in FN coated (FN-adherent group) or uncoated (FN-adherent group) petri dishes and incubated at 37°C for 24- hour. Tu (24 µM), Cp (50 µM) and Tu- Cp were applied to the cells for 1- and 12-hours before the end of the incubation period. The cells lysed after the incubation. The amount of protein in cell lysates was determined with Bradford method. Equal amounts of protein (50µg) samples were loaded on 10% acrylamide gel (29:1 w/w acrylamide:bis-acrylamide) and separated into protein fractions, then transferred to the polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% BSA, and incubated with primary antibodies p-FAK (sc-374668), Rac Family Small GTPase 1 (Rac1) (ab33186), p-Src (sc-81521), Proline-rich tyrosine kinase 2 (Pyk2) (sc-393181), Cell Division Cycle 42 (Cdc42) (Thermo fisher, PA1-092), and actin (sc-7210) at 4°C overnight. The membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (sc-2005) or anti-goat IgG (sc-2004) secondary antibodies for 1 hour at room temperature (21^0C), and exposed to 3,3′,5,5′-Tetramethylbenzidine (T0565, Sigma-Aldrich) until bands appear on it. Finally, after washing with deionized water, protein bands were visualized using the UVP Bio spectrum 510 Imaging System.

Statistical analysis

Cell index values obtained from cell migration assay with RTCA device were evaluated using Student's t-test. The statistical tests were conducted by using STATA 18 (***$P \leq 0.01$; **$P \leq 0.05$; *$P \leq 0.10$).

Results

Cell migration

The effect of Tu, Cp and Tu-Cp on cell migration in FN-adherent/non-adherent IHOSE-SV40 and SKOV-3 cells was analyzed with Xcellingence for 24hours. Cell migration data obtained at 12- and 24-hourswere analyzed by t-test (Fig. 1 &Fig. 2). The results showed that FN-adherent normal cells migrated at a high rate (cell migration index mean: 21.91) at 24hours (Fig. 1A). Administrations of Tu (12-h, P=0.011; 24-h, P<0.001), Cp (12-h: P=0.024, 24-h: P= 0.022) and Tu-Cp (12-h: P=0.004, 24-h: P=0.003) led to inhibition of cell migration significantly at both 12 and 24 hours in FN-adherent IHOSE-SV40 cells. In addition, Tu-Cp administration in FN-adherent IHOSE-SV40 cells inhibited cell migration more strongly at 12hours than Tu (P=0.023) and Cp (P=0.062) groups, and at 24hours compared to Tu group (P=0.039). Compared to single applications of Tu (12-h: 39 %, 24-h: 49%) and Cp (12-h: 35 %, 24-h: 47%), combination of Tu and Cp inhibited 80% at 12 hours and 86% at 24hours, and produced the strongest inhibition in FN-bound IHOSE-SV40 cells. We observed that the migration index in FN-non-adherent IHOSE-SV40 cells compared to FN-adherent cells was very low both in

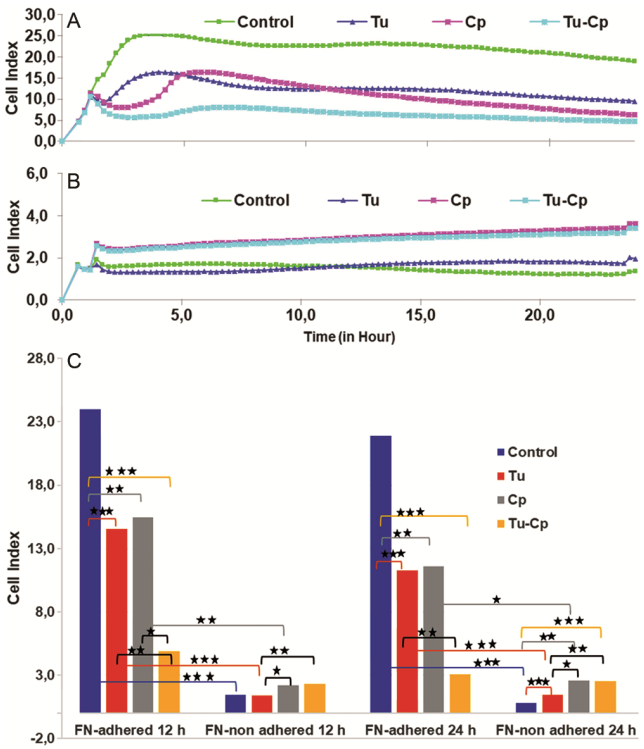

Fig. 1 — The effect of Tu, Cp and Tu-Cp treatments on cell migration in FN-adherent/non-adherent IHOSE-SV40 cells. (A) Time-dependent change of cell migration index in FN-adherent. (B) Time-dependent change of cell migration index in FN-non-adherent IHOSE-SV40 cells for 24- hour. One of the two independent experiments is shown in A and B. In the graphs, each experimental group was drawn by taking the average of two wells (n=2). (C) Cell migration rate and statistical result after 12- and 24-hours in IHOSE-SV40 cells (n=4). The cell index values in the graph presented are the means of two independent experiments (n=4). Control: No treatment, Tu: Tu applied cells, Cp: Cp applied cells, Tu-Cp: Tu and Cp applied cells. (*** $P \leq 0.01$, ** $P \leq 0.05$, * $P \leq 0.10$).

the control and the treatment groups (Fig. 1B). While Tu, Cp and Tu-Cp treatments in FN-non-adherent IHOSE-SV40 cells did not show a significant change at 12 hours compared to the control group, they stimulated cell migrationsignificantly by Tu: 79%, Cp: 215%, Tu-Cp: 213% at 24hours (Fig 1C).In FN-non-adherent IHOSE-SV40 cells, both 12 hours (P=0.015) and 24 hours (P=0.022) Tu-Cp treatments stimulated cell migration more than Tu administration.

The average 24 hours migration index for FN-adherent SKOV-3 cells was calculated to be 14.75 (n=4) (Fig. 2A). Unlike the FN-non-adherent IHOSE-SV40 cells, the cell migration index in both the control and treatment groups of FN-non-adherent SKOV-3 cells showed a negative value, indicating a lack of cell migration (Fig. 2B). Similar to the result of FN-adherent IHOSE-SV40 cells, Tu (12-h and 24-h: P <0.001), Cp (12-h: P=0.098, 24-h: P<0.001), and Tu-Cp (12-h and 24-h: P<0.001) applications inhibited cell migration significantly in FN-adherent cancer cells at both 12- and 24-hours (Fig. 2C). In FN-adherent SKOV-3 cells, both 12- and 24-hoursTu application was more inhibiting the cell migration compared to both Cp (12-h: P=0.017, 24-h: P=0.001), and Tu-Cp (12-h: P=0.002, 24-h: P=0.017) applications.Tu-Cp application was more inhibitive of cell migration than Cp (12-h: P=0.028, 24-h:P=0.005) application. When Cp (12-h: 20%, 24-h: 59%) and Tu-Cp (12-h: 74%, 24-h: 91%) applications were compared with Tu, Tu was found to be the most potent inhibitor of cell migration in the FN-bound SKOV-3 cell group, with 85% inhibition at 12 hours and 99% at 24hours.

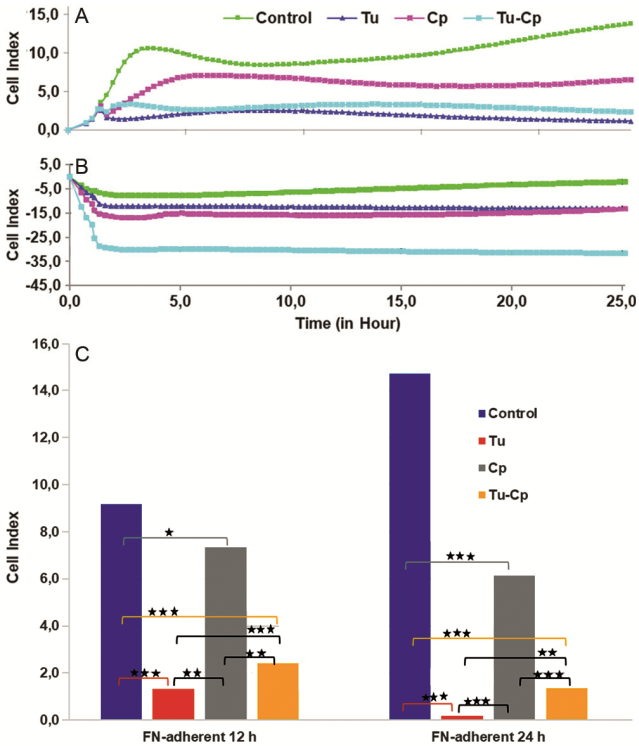

Fig. 2 — The effect of Tu, Cp and Tu-Cp treatments on migration in FN-adherent/non-adherent SKOV-3 cells. (A) Time-dependent change of cell migration index in FN-adherent. (B) Time-dependent change of cell migration index in FN-non-adherent SKOV-3 cells for 24- hour. One of the two independent experiments is shown in A and B. In the graphs, each experimental group was drawn by taking the average of two wells (n=2). (C) Cell migration rate and statistical result after 12- and 24- hour in SKOV-3 cells (n=4). The cell index values in the graph presented are the means of two independent experiments (n=4). Control: No treatment, Tu: Tu applied cells, Cp: Cp applied cells, Tu-Cp: Tu and Cp applied cells. (*** P≤0.01, ** P≤0.05, * P≤0.10).

Localization

The localization of p-FAK, p-paxillin and vinculin, could not be detected in FN- adherent/non-adherent IHOSE-SV40 cellsafter 1-hour of incubation.After 12hours of incubation, p-paxillin localization was observed in the FN-adherent control and Tu groups (Fig. 3A), and vinculin in FN-adherent groups (Fig. 3B), while p-FAK localization was not observed.In Fig. 3B, accumulation of vinculin at focal adhesion sites which are important for mesenchymal type of migration was observed in FN-adherent control vinculin group, whereas typical cytoplasmic staining of vinculin was observed in cells treated with Tu, Cp or their combination. Similar to the findings in IHOSE-SV40 cells, the localization of vinculin was not observed in SKOV-3 cells after a 1-hour incubation period. However, in SKOV-3 cancer cells, a distinct pattern emerged where p-paxillin localization was evident in both the FN-adherent Tu and Tu-Cp groups (Fig. 4A), while p-FAK localization was observed in FN-adherent groups (Fig. 4B), contrasting with the normal cell results at the same 1-hour incubation time. At 12hours incubation, p-paxillin and vinculin localization was observed in all FN- adherent/non-adherent groups except FN-non-adherent Tu-Cpgroup (Fig. 5 & Fig. 6), p-FAK localization could not be detected.

Protein expressions

Expressions of p-FAK, Pyk2, p-Src, Cdc42 and Rac1 proteins in FN- adherent/non-adherent IHOSE-SV40 cells were investigated by western blot method after 1- and 12-hours of Tu, Cp and Tu-Cp treatments (Fig. 7A& Fig. 7B). Actin protein expression served as the control in this study.In addition, densitometric

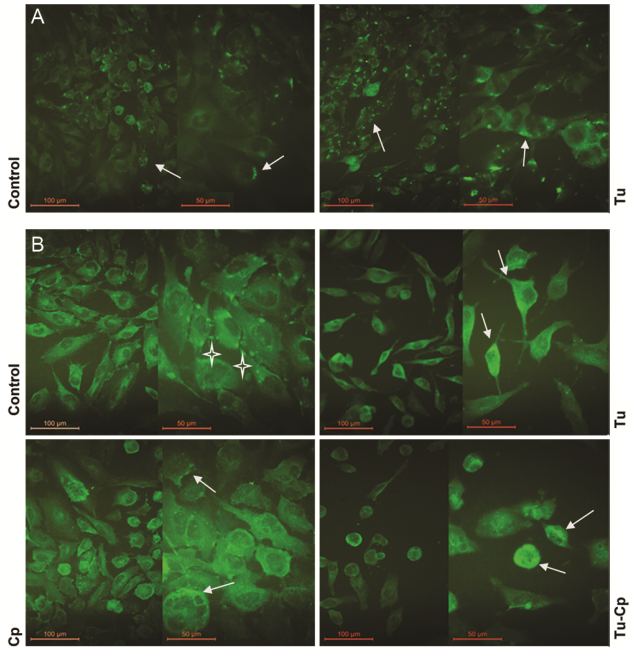

Fig. 3 — Localization of p-paxillin and vinculin in FN-adherent IHOSE-SV40 cells after 12- hour of administration. (A) P-paxillin localization. (B) Vinculin localization. →; protein localization (bright green), vinculin accumulation at focal adhesion sites. Control: No treatment, Tu: Tu applied cells, Cp: Cp applied cells, Tu-Cp: Tu and Cp applied cells.

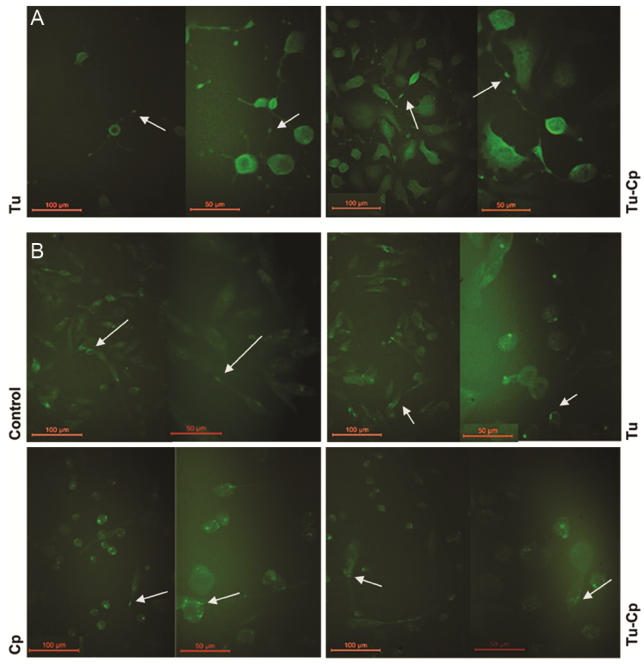

Fig. 4 — Localization of p-paxillin and p-FAK in FN-adherent SKOV-3 cells after 1 hour of administration. (A) P-paxillin localization. (B) p-FAK localization. →; protein localization (bright green). Control: No treatment, Tu: Tu applied cells, Cp: Cp applied cells, Tu-Cp: Tu and Cp applied cells.

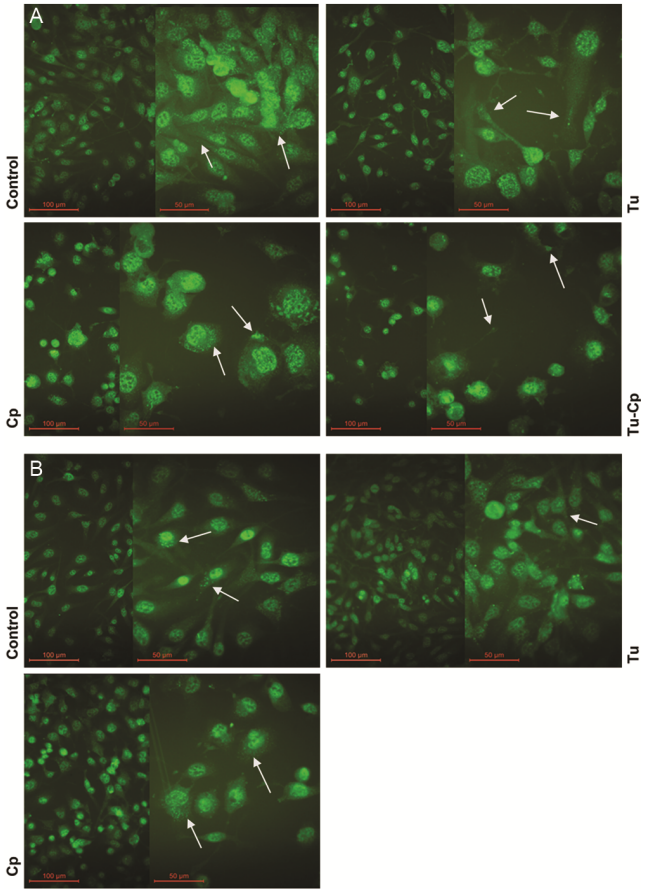

Fig. 5 — Localization of p-paxillin in FN-adherent/non-adherent SKOV-3 cells after 12- hour of administration. (A) P-paxillin localization in FN-adherent cells. (B) P-paxillin localization in FN-non-adherent cells. →; protein localization (bright green). Control: No treatment, Tu: Tu applied cells, Cp: Cp applied cells, Tu-Cp: Tu and Cp applied cells.

analysis of protein bands was performed (Fig. 7C).After 1-and 12- hour of Tu, Cp and Tu-Cp administration in IHOSE-SV40 cells, p-FAK protein was observed to be expressedlow in all groups and its expression increased in FN-adherent groups. Tu and Cp treatments increased p-FAK expression at 1 hour and reduced at 12 hours in FN-adherent groups. On the other hand, treatment of Tu-Cp increased p-FAK expression in both at 1- and 12- hours in FN-adherent groups. Tu, Cp and Tu-Cp treatments decreased p-FAK expression in FN-non-adherent group at 12 hours.Pyk2 expression was detected at low levels at both 1- and 12-hours across all experimental groups.Pyk2 was more expressed in FN-non-adherent control groups than FN-adherent control groups.In FN-non-adherent groups, Cp treatment increased Pyk2 expression while Tu and Tu-Cp treatments decreased. P-Src expression was evident at 1 hour but

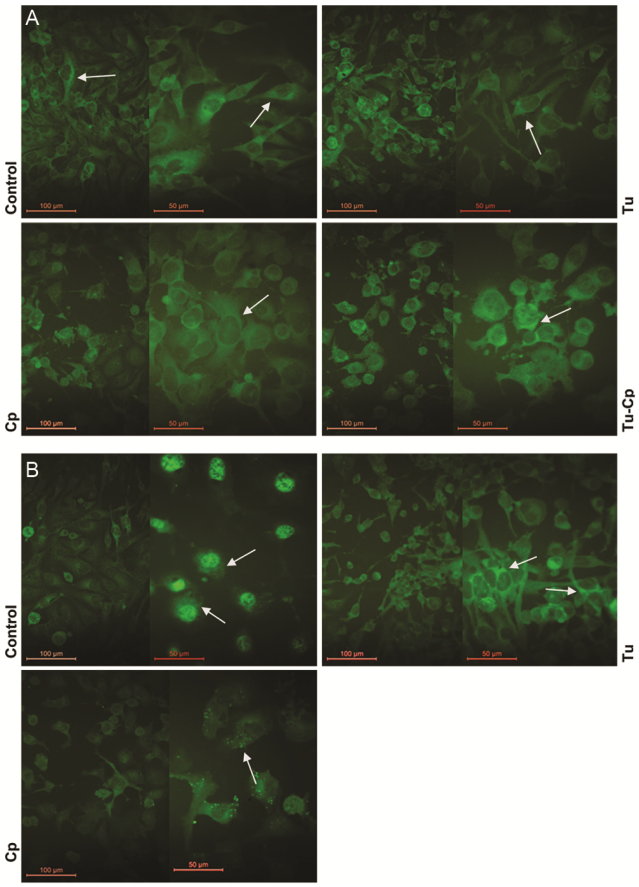

Fig. 6 — Localization of vinculin in FN-adherent/non-adherent SKOV-3 cells after 12- hour of administration. (A) Vinculin localization in FN-adherent cells (B) Vinculin localization in FN-non-adherent cells →; protein localization (bright green). Control: No treatment, Tu: Tu applied cells, Cp: Cp applied cells, Tu-Cp: Tu and Cp applied cells.

absent at 12 hours. Cp application increased p-Src expression more than2.5-fold in FN-non-adherent group at 1 hour. Cdc42 protein expression was observed both at 1 and 12-hour. 1-hour Tu administration decreased Cdc-42 expression in the FN adherent group. On the other hand, at 12 hours, Tu, Cp and Tu-Cp applications all increased Cdc42 expression in the FN adherent group. In the FN-non-adherent group, Tu, Cp and Tu-Cp applications increased Cdc42 expression in 1 hour and decreased it at 12- hour.Rac1 protein showed significant levels of expression at both 1- and 12-hour, irrespective of the experimental conditions. At 1-hour incubation period, Cp and Tu treatments decreased Rac1 expression in both FN-adherent and FN-non-adherent groups. However, unlike Cp and Tu, in the FN-adherent group, the combination treatment of Tu-Cp increased Rac1 expression at both 1 and 12-hour of

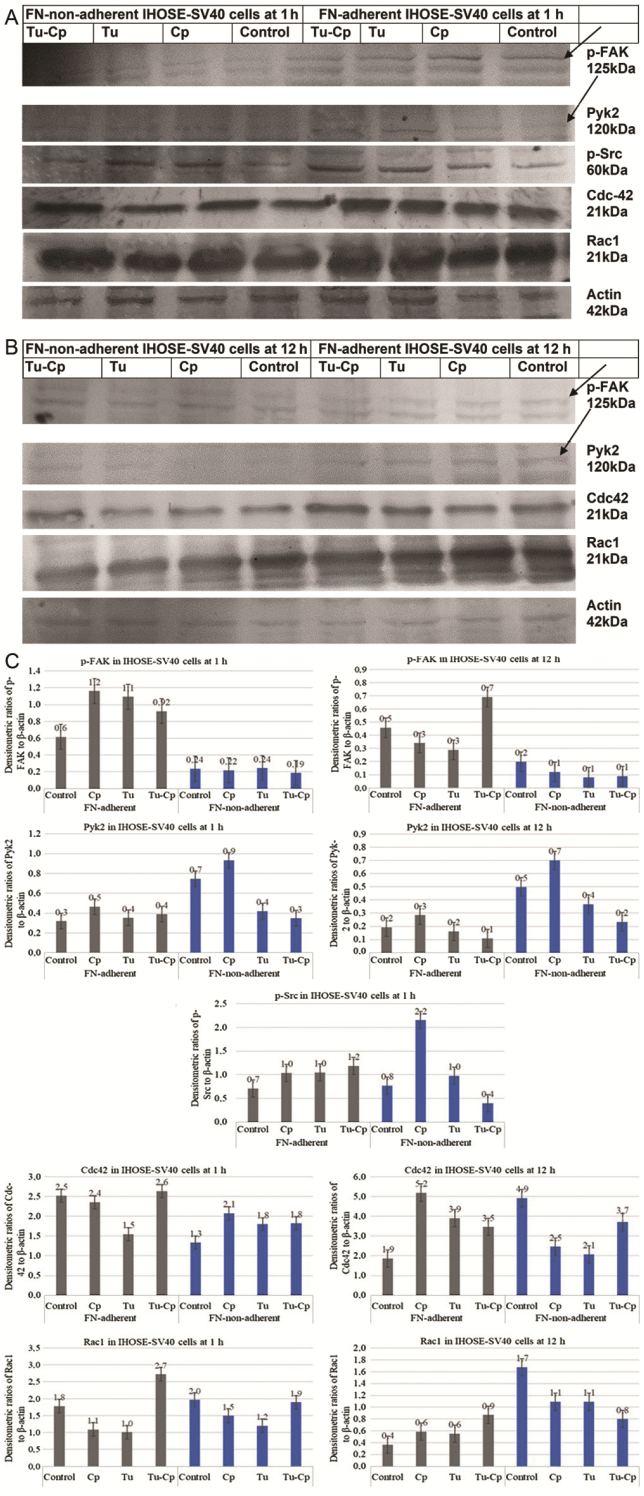

Fig. 7 — Protein expressions in FN-adherent/non-adherent IHOSE-SV40 cells. (A) 1 hour incubation group. (B) 12- hour incubation group. (C) Densitometric analysis of target protein/β-actin.Control: No treatment, Tu: Tu applied cells, Cp: Cp applied cells, Tu-Cp: Tu and Cp applied cells.

administration. At 12 hours application, Cp, Tu and Tu-Cp all increased Rac1 expression in the FN-adherent group and decreased it in FN-non-adherent group.

Expression levels of the proteins were determined after the incubation ofTu, Cp or Tu-Cpin SKOV-3 cells at 1- and 12-hour(Fig. 8A& Fig. 8B). Densitometric analysis of protein bands is provided in (Fig. 8C). p-Fak protein expression was not observed after 1 hour of application. After 12 hours of incubation, however, it was observed in all groups except FN-non-adherent Tu-Cp group. p-FAK was expressed more in FN-adherent groups except the control group. Additionally, Cp, Tu and Tu-Cp applications increased p-FAK expression in FN-adherent groups while reduced the expression in FN-non-adherent groups.During the incubation period, Pyk2 protein expression was not observed. In the groups subject to1- and 12-hour applications, the expression of p-Src protein was observed in all groups. After 12 hours of application, p-Src expression was expressed more in FN-adherent groups than in FN-non-adherent groups.Cp (12 h), Tu (1 and 12 h) and Tu-Cp (1 h) applications reduced p-Src expression in FN-adherent groups while increased the expression in FN-non-adherent groups (12h).Cdc42 and Rac1 expressions were observedat high levels at both incubation periods. Cp application caused a decrease in Cdc42 expression in FN-adherent/non-adherent groups in both incubation periods. Tu application decreased Cdc42 expression in FN-non-adherent groups after 1 hour of application while it increased it after 12 hours of application. Tu-Cp application increased Cdc42 expression in FN-adherent groups at both time periods.While 1hourCp application decreased Rac1 expression in FN-adherent group, it increased Rac1 expression in non-FN-adherent group. Tu application, however, increased Rac1 expression in FN-adherent/non-adherent groups. Tu-Cp application did not cause any significant change in FN-adherent group, but increased in non-FN-adherent group. At 12 hours, Rac1 expression was higher in FN-adherent group than FN-non adherent group. Tu and Tu-Cp treatments increased Rac1 expression in both FN-adherent/non-adherent groups.

Discussion

Fibronectin, an extracellular matrix protein (ECM), has many important functions in cell differentiation, adhesion, and migration[20]. FN expression is prevalent in various cancer types and plays a role in tumor initiation and metastasis[21]. The presence of FN in ovarian cancer holds prognostic value. Elevated level of FN in the tumor stroma is strongly linked to poorer overall survival outcomes. Furthermore, metastatic lesions exhibit increased FN expression compared to the primary tumor[22]. In this study, we found that while ovarian cancer cells must be bound to FN to migrate, normal ovarian cells can migrate without binding to FN. Migratory abilities of chemoresistance ovarian cancer can increase through activation of calpain by upregulation of store-operated Ca^{2+} entry. Cp markedly suppressed the calpain-induced cell migration during the wound healing process in chemoresistant ovarian carcinoma (IGROV1) cells[23]. We showed that Cp, Tu and Tu-Cp applications significantly inhibited cell migration in FN bound both normal ovarian and ovarian cancer cells at 24 hours. In addition, we determined in FN-bound SKOV-3 cells that Tu (99%) administration inhibited cell migration more significantly than both Cp (59%) and Tu-Cp (91%) administration. Tu-Cp administration also inhibited significantly more than Cp administration. The combined application of Tu and Cp did not have a better effect than the application of Tu alone at 24- hour. In FN-bound normal, Tu application inhibited by 49%, Cp application by 47%, and Tu and Cp combined application inhibited by 86%. In contrast to cancer cells, Tu and Cp combined application led the highest inhibition in normal cells. In order to gain deeper insights into the impact of Tu, Cp, and Tu-Cp on migration in FN-bound ovarian cancer cells, it is essential to explore their influence on proliferation and apoptotic mechanisms. Research findings regarding Tu have indicated its capability to suppress cell migration in hepatocellular carcinoma[15]. In addition,Tu treatment induced autophagy and apoptosis while inhibiting proliferation in ovarian cancer SKOV3 cells[24]. Studies have also shown that Tu triggered apoptosis and suppressed the Wnt/β-catenin pathway, leading to a reduction in cell viability in breast cancer cells[25]. Cphas been reported to inhibit cell invasion in renal cell carcinoma ACHN cells[26]. In research on the apoptotic effect of Cp, it has been reported that Cp inhibits apoptosis in melanoma cells[27]. Zeng reported that Cp protects myoblasts from apoptosis by inhibiting mitochondrial calpain activity in a myoblast-carcinoma cell coculture model[18].

FA, which is involved in cell migration, is the structural and signal junction complex between the

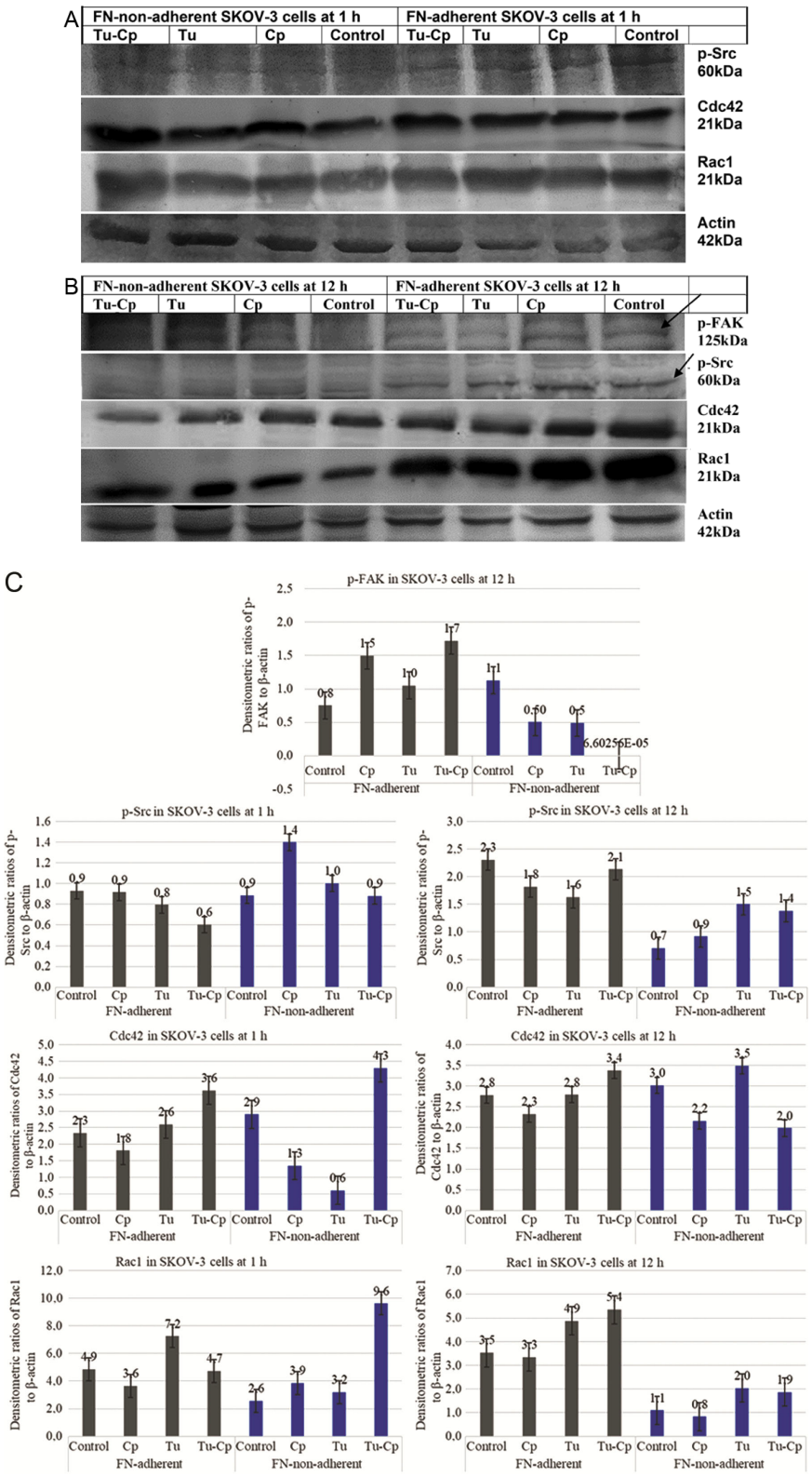

Fig. 8 — Protein expressions in FN-adherent/non-adherent SKOV-3 cells. (A) 1 hour incubation group. (B) 12- hour incubation group (C) Densitometric analysis of target protein/β-actin.Control: No treatment, Tu: Tu applied cells, Cp: Cp applied cells, Tu-Cp: Tu and Cp applied cells.

ECM and the actin cytoskeleton. The rate of cell migration is regulated by the formation and degradation of FA[28]. Calpains are involved in the turnover of FA during cell migration, specifically by promoting the proteolysis of FA proteins like FAK, Paxillin, and Vinculin at the trailing edge[29]. In this study, we investigated cytoplasmic localizations of p-FAK, vinculin and p-paxillin. Detection of p-FAK localization in FN-adherent SKOV-3 cells, not in IHOSE-SV40 cells, shows that p-FAK localization has an important role in the migration of ovarian cancer cells. At 12 hours of incubation, vinculin and p-paxillin (Tyr 118 phosphorylated) localizations were observed in FN-adherent/non-adherent SKOV-3 cell groups (except FN-non-adherent Tu-Cp group). On the other hand, in IHOSE-SV40 cells, while vinculin was observed in FN-adherent groups, paxillin was observed only in FN-adherent Tu group. In the IHOSE-SV40 cell group, vinculin was found to accumulate at FA regions. However, FA regions of cells treated with Tu, Cp, and Tu-Cp showed no signs of vinculin accumulation. Consequently, treatment with Tu, Cp, and Tu-Cp effectively hindered the accumulation of vinculin at focal adhesion sites in FN-adherent IHOSE-SV40 cells. Furthermore, Tu treatment prompted the localization of p-paxillin in the cytoplasm of both FN-bound IHOSE-SV40 and SKOV-3 cells at various time intervals. Paxillin is the substrate of the Src-FAK complex and tyrosine residues phosphorylated paxillin is essential for FA disassembly. In cell migration, FAK and paxillin are involved in FA assembly and disassembly. FAK assembles before paxillin in the newly formed FA in front of the cell[30].

Cells communicate with Fibronectin via integrin, promoting cellular migration and cell differentiation[31]. Activated by integrins, FAK is engaged in various cellular functions such as cell proliferation, survival, motility, and embryonic development. FAK collaborates with Pyk2 to carry out specific cellular activities. In the context of cancer, FAK plays a crucial role in facilitating tumor growth and metastasis[32]. It has been reported that ovarian cancer cells treated with FN may have an increased ability to migrate and invade through phosphorylation of FAK[33]. Cell migration relies significantly on the FAK protein, which plays a crucial role in controlling focal adhesion sites and guiding the orientation of cellular movement[19]. Studies have shown that FAK, which is strongly associated with tumor formation and development, is highly expressed in many cancer cells[34]. In this study, higher expression was observed in both FN-adherent and FN-non-adherent untreated ovarian cancer cells. Although we detected P-FAK localization in cancer cells, it was not detected in normal cells, which may be related to lower expression in normal cells.

The biochemical link between intracellular Ca^{2+} and FA in cell migration is provided by the Pyk2 protein localized to the FA. This binding protein, Pyk2, is activated in a Ca^{2+}-dependent manner[35]. Activation of calpain and Pyk2 regulates several signaling pathways necessary for FA formation, disassembly, and cell migration[28]. The store-operated Ca (2+) entry Pyk2 pathway has been reported to play a critical role for glioma migration and invasion[36]. Pyk2 protein expression was observed in IHOSE-SV40 cells, though not observed in SKOV-3 cells.

While the exact mechanism of its impact remains unclear, the Src protein plays a role in regulating normal cell processes like cell growth, division, and viability. Src expression and activity are increased in various human cancers and have been found to be associated with metastasis[37]. The FAK protein is activated by autophosphorylation from the tyrosine region in the cell migration mechanism and interacts with Src family kinases and takes part in the formation of many signal complexes[38]. In our study, while p-Src expression was observed in both cell lines in 1 hour application, it was not observed in IHOSE-SV40 cells with the increase of the duration to 12-hour. Moreover, we observed p-Src expression increase in SKOV-3 cells (12 h) adhering to FN.Cortesio et al. reported that there is a new signaling pathway including calpain 2, protein tyrosine phosphatase 1B, and Src in the regulation of invadopodia and breast cancer invasion[39]. In their research on hepatocellular carcinoma, Dai et al. found that CAPN-4, identified as the regulatory component of CAPN-1/2, influences tumor progression and spread through the activation of the FAK-Src signaling pathway[40]. In our study, FAK and p-Src expression was observed in both normal and ovarian cancer cells. With FN binding, we found that p-FAK expression in IHOSE-SV40 and p-Src expression in SKOV-3 cell were increased.Tu and Cp treatments decreased p-FAK expression in FN-adherent/non-adherent normal cells. Tu-Cp treatment down regulated p-Src expression in FN-non-adherent group.In ovarian cancer cells, Cp, Tu and Tu-Cp applications decreased p-FAK expression in FN-non-adherent groups and p-Src expression in FN-adherent groups.

Rac and Cdc42 are involved in important tasks such as regulating cell division and actin cytoskeleton rearrangements, cell migration/invasion, polarity, and adhesion. The activation of Rac/Cdc42 has been linked to the development of metastatic cancers. Thus, targeting the activation of Rac/Cdc42 shows promise as a potential therapy for metastatic cancer[41]. When we examined the expressions of Rac1 and Cdc42 proteins in SKOV-3 and IHOSE-SV40 cells, we observed Rac1 and Cdc42 proteins in both cell lines in both time periods. In addition, Rac1 expression was increased in SKOV-3 cellsadhered to FN. In SKOV-3 cells, the expression of p-Src was detected in all experimental groups after 1 and 12-hour. At 12 hours, we found that p-Src expression increased by binding of the cells to FN.Overall, the levels of p-Src, Cdc42, and Rac1 were found to be elevated in FN-adherent SKOV-3 cells at the 12hours.

Consequently, there is a potential correlation between the p-Src, Cdc42, and Rac1 signaling pathways in SKOV-3 cells. Previous research has indicated that FAK/c-Src signaling complex activates Rac1 and leads to the phosphorylation of the cytoskeletal protein p130 Cas. Additionally, it has been documented that the Rac1 and Cdc42 GTPase proteins play a role in regulating the assembly of focal complexes crucial for cell migration[38]. Importantly, the Cp, Tu, and Tu-Cp treatments applied in this study reduced the expression of these three signaling proteins (Cdc42, p-Src, and Rac1).

Conclusions

Our study demonstrated that while normal cells can undergo migration independently of fibronectin binding, ovarian cancer cells depend on fibronectin binding for their migratory capabilities. FN triggers distinct signaling pathways in normal cells (p-FAK) and ovarian cancer cells (p-Src and Rac1). We show that, treatments with Tu, Cp and Tu-Cp significantly blocked cell migration in both FN-bound normal and cancer cells in this study. Specifically, our results indicated that Tu was the strongest inhibitor of migration in FN-bound ovarian cancer cells. These treatments were also observed to have down-regulatingeffects on the expression of molecules involved in migration pathways. In FN-dependent cancer cells, however,Tu and Tu-Cp treatments only reduced p-Src expressionwhile Cp treatment down-regulated p-Src, Cdc42 and Rac1 expressions.

As a result, this study suggests that Cp may be a new agent in the treatment of ovarian cancer due to its inhibitory effect on migration and down regulatory effect on Rac1/Cdc42 proteins. The results of this study will contribute to the understanding of the molecular mechanism of calpeptin, specific calpain inhibitor, and calpain in ovarian cancer. It will also contribute to the understanding of the molecular mechanism of tunicamycin shown as a potential anti-cancer agent. However, since *in vitro* studies do not fully reflect the tumor microenvironment, further *in vivo* studies are needed.

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Conflict of Interest

The author declares no conflict of interest.

Ethics Approval and Consent to Participate

This article does not contain any studies involving human participants or animals performed by any of the authors.

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