The Ca^{2+} mobilisation and the inhibition of akt reduced the binding of PEO-1 cells to fibronectin

Introduction

The cell adhesion molecules, integrins, activate outside-in signalling pathway necessary for cell invasion by mediating attachment between cells and extracellular matrix (ECM). The integrins participate in cell morphology, migration, proliferation, differentiation, survival (1-2) as well as apoptosis. Integrin-mediated cell attachment is necessary to cancer cell metastasis and invasion. Moreover, ECM-integrin signalling is said to have survival advantage in different types of cancer cell against many chemotherapeutic treatments (2). The studies on this topic are usually based on integrin expressions. Signal transduction mechanisms of integrins in ovarian cells, however, have not been explicitly investigated yet.

Cell-ECM adhesiveness is generally increased in human cancers that allows the cancer cells to destroy the histological structure. Reduced the cell-ECM adhesiveness is also important for cancer invasion and metastasis (3). Moreover, some studies showed that the adhesion capacity of high metastatic cells could be reduced by inhibiton of integrin expression or varies process that leads to metastatic cells to anoikis which is a programmed cell death induced upon cell detachment from ECM (4-5). Especially, the αv integrin subunit-inhibiton or αv-knockdown inhibited the colonogenic and migratory ability of human prostate cancer cells *in vitro* and *in vivo* (4,6). αv integrin is, thereby, said to be a promising target for cancer therapy strategies.

Ovarium cancer cells can spread as a cell form or spheral form from the surface of ovarium. Therefore, metastatic ovarium cells should survive and proliferate without ECM binding. The microenvironment of cells is dynamic and containing survival factors such as cytokines, growth factors, hormones, proteases and ECM proteins that regulate tumor cell migration, invasion, survival and spheral forms (7). In particular, fibronectin and vitronectin (ECM proteins) proteins induce the formation of spheroids, adherence and disaggregation of ovarium cancer cells. These proteins disintegrated by metalloproteinase-2 increase the adhesion of ovarium cancer cells to region of peritoneal and that is nascent stage of metastasis (8).

β-catenin is a multi-functional protein and involve in WNT signal pathway as well as adhesion via E-cadherin in epithelial cells (9). In normal epithelial cells, β-catenin binds to E-cadherin- α-catenin complex in adherent junctions., In the presence of WNT signalling, however, β-catenin accumulates in the cytoplasm and then translocate to the nucleus due to activation of large number of target genes included LEF/TCF genes (10). These activated genes are attributed to the development of some diseases, especially various types of human cancers. A number of studies show that accumulation of β-catenin is also effective in creating a suitable microenvironment for cancer progression (11-12).

Recently, it has been shown that Akt is one of the most effective regulatory proteins in β-catenin accumulation process. Especially, N-cadherin adhesion can lead to phosphatidylinositide 3-kinases (PI3K) mediated activation of Akt and that might stimulate β-catenin signalling pathway (13). Moreover, Akt protein also phosphorylates GSK3β and lead to inactivate the function of GSK3β. In this case, stabilization and accumulation of β-catenin is induced (14).

The current study aims to investigate the role of Ca^{+2} increasing via tunicamycin (TN) treatment and β-catenin-Akt signaling on the binding of metastatic ovarian cancer cell (PEO-1) to fibronectin. Here we investigated the expression levels of integrins that play an active role in PEO-1 binding to fibronectin by flow cytometry and immunofluorescence staining. Using aRTCA), we show that increasing of cytoplasmic calcium in PEO-1 cells influenced the cells adhesion. Inhibition of accumulation of β-catenin and Akt signalling using specific inhibitors lead to inhibition of PEO-1 adhesion to fibronectin. These results suggest a link between adhesion of PEO-1 ovarian cells and Ca^{2+} mobilisation, function of Akt and β-catenin.

Materials and Methods

Cell culture

The PEO-1 human ovarian cancer cell line was purchased from Public Health England (10032308) and cultured in RPMI 1640, 10 % Foetal Bovine Serum, 2 mM sodium Pyruvate and 2 mM Glutamine.

Detection of integrin expression

Expression levels of αv, α4, β1 and β6 integrin were determined using specific antibodies by flow cytometry on PEO-1 cells. The cells were incubated with 1:200 dilution of primary antibodies against integrin subunits, subsequently washed in PBS and incubated with 1:200 FITC-conjugated secondary antibody for 30 min at 4°C. Control cells contain either primary antibody or FITC secondary antibody. After washing, all samples were analysed using flow cytometer (Becton Dickinson, FACSAria II, Canada).

Localisation of integrins on cell membrane

The localisation of integrins was detected by using florescence microscopy. Coverslips were coated with 50 µg/ml fibronectin. The cells were then seeded, washed with PBS and fixed with 4 % formaldehyde, washed again and then permeabilized with 0.1 % Tween-20. After washing with PBS, the cells were treated with 1 % Bovine Serum Albumin (BSA). The cells were treated with specific primary integrin antibodies (1:200 dilution) for overnight at +4 °C, then FITC- conjugated secondary antibody (1:300 dilution) for 1 h at +4°C. Primary antibody was not added in the control group. After washing the coverslips , they were mounted on microscope slides. The slides were finally examined by using florescence microscopy and monitored.

Binding Assays

The binding rate of PEO-1 cells to fibronectin

The RTCA was used for real-time and time-dependent analysis of binding of PEO-1. The RTCA measure changes in electrical impedance as cell attach and spread in a multi-well plates covered with a gold microelectrode array (15). The electrode impedance is displayed and recorded as “cell index” values that reflect the biological statue of monitored cells, including the cell number, cell viability, morphology and cell adhesion (16).

16-well plates were coated with different concentration (1 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml) of fibronectin except control wells. The wells were washed with PBS and then blocked with 1 % BSA for inhibiting non–specific binding. After a background impedance measurement, 5 $\times 10^4$ cell/well were seeded. The impedance was monitored at 15 min interval during 24 h. The rate of cell

adhesion was calculated according to normalize the cell index (NCI) or cell index formula.

NCI = (impedance at time point n – impedance in the absence of cells) / nominal impedance value.

CI = (resistance measured at a time point – resistance measured without the cell) / 15Ω

The Role of $[Ca^{+2}]i$ on PEO-1 Cell Adhesion Capacity

Integrins on cell surface were activated by fibronectin (50 µg/ml). The effect of $[Ca^{+2}]i$ on cell adhesion capacity was investigated by the RTCA system after stimulated PEO-1 cells by using IP_3-independent intracellular calcium release, thapsigargin (TG) (50 nM, 100 nM, 500 nM, 1 µM, 2 µM) and extracellular calcium channel blocker TN (0,5-1-5-10-20 µg/ml). PEO-1 cells were treated with 5, 20, 35 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) which is Ca^{+2} chelator to create a non-calcium environment. Measurement of cell adhesion was conducted for 24 h.

The Role of Cardamonin and Akt on Binding Ability of PEO-1 cells

16-well plates were prepared as mentioned above. After the wells were coated with 50 µg/ml fibronectin, the plates were seeded with 5×10^4 PEO-1 cells and transferred into RTCA for 2 h incubation before monitoring. After that, the cells were treated with different concentrations of cardamonin (3,13 µM, 6,25 µM, 12,5 µM, 25 µM, 50 µM, 100 µM), an inhibitor β-catenin, and FPA 124 (5 µM, 15 µM, 25 µM, 35 µM), Akt signal pathway inhibitor. The cells were monitored at 30-min intervals for a period of 24 h. Uncoated wells were used as a control groups together with the wells containing no inhibitor.

Results

Expression and Localisation of Integrins on the Surface of PEO-1 cells

The expression and localisation of α4, β1, αv and β6 integrin subunits were investigated by using flow cytometry and immunofluorescence staining. The results of flow cytometric analysis were given in Figure 1. The expression levels of αv, α4, β6

and β1 were ordered descendingly with respect to expression level. The expression level of αv integrin subunit is found to be abundant compared with the others.

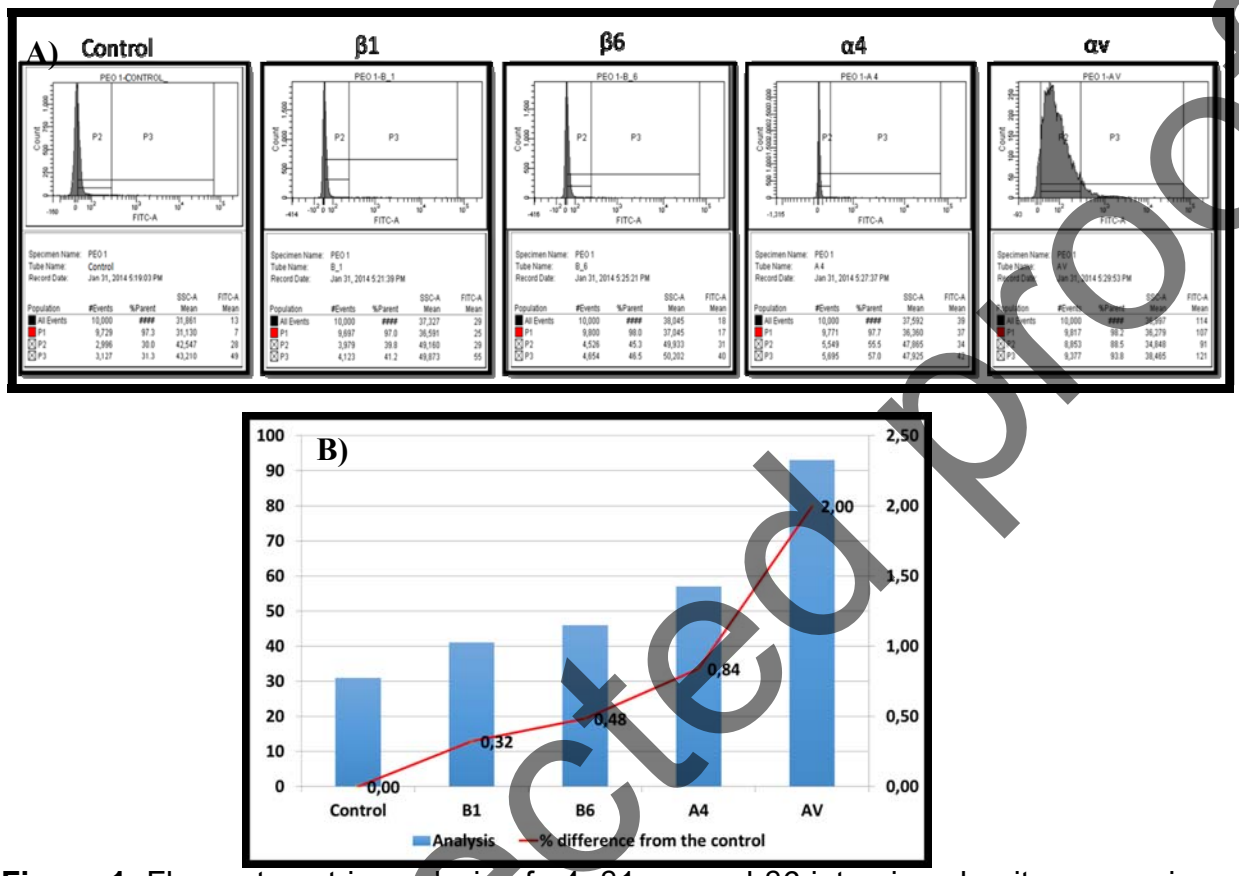

Figure 1. Flow cytometric analysis of α4, β1, αv and β6 integrin subunits expression **(A)** and percentage of integrin subunits expressions **(B)** on the surface of human ovarian cancer PEO-1 cell line. **(A)** Cells first were treated with α4, β1, αv and β6 primary antibodies except control, and then FITC conjugated secondary antibodies. **(B)** Left axis is expression level, right axis is the percentage differences from the control group. Expression levels of α4, β1, αv and β6 are higher about 32 %, 48 %, 84 %, and 200 % than the control group respectively.

PEO-1 cells were incubated with specific primary integrin antibodies and localisation of integrin subunits was demonstrated by fluorescence microscopy (Figure 2). PEO-1 cells showed low but detectable level of β6 integrin subunit localisation whereas cells exhibited considerable amounts of α4, αv and β1 localisations over the whole cell as labelled with specific antibodies.

The Binding Rate of PEO-1 Cells to Fibronectin

PEO-1 cells were examined by RTCA system to determine whether the binding of cells to various concentrations (1-100μg/ml) of fibronectin. The results, shown in Figure 3, indicate that PEO-1 cells bind to fibronectin at all concentrations compared

to control cells. The cells showed a higher percentage binding to 50 µg/ml fibronectin (the cell index 5-6 at 24 h), however while the low fibronectin concentration (1 µg/ml) supported little cell adhesion. The result suggests that 50 µg/ml fibronectin seems to be a suitable concentration for using further studies.

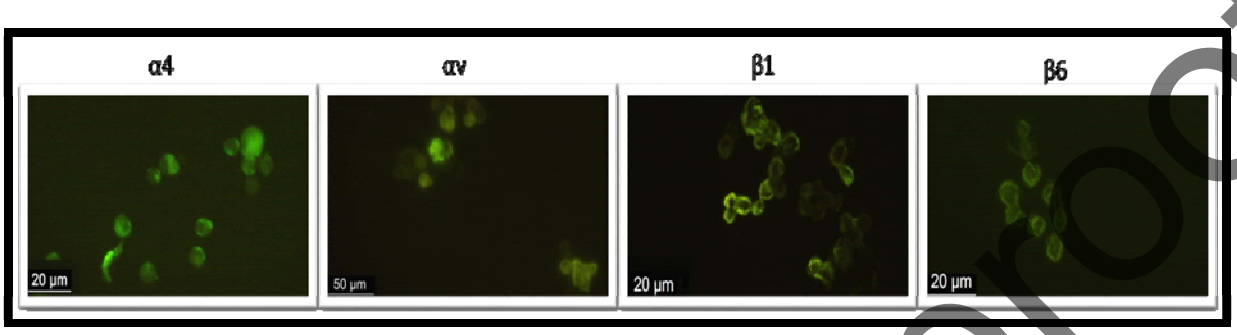

Figure 2. Immunofluorescence staining of human ovarian cancer PEO-1 cells for α4, αv, β1 and β6 integrin subunits. The cells were labelled with various primary integrin antibodies at 37°C for 1 h. After that, PEO-1 cells were incubated with FITC-conjugated secondary antibody.

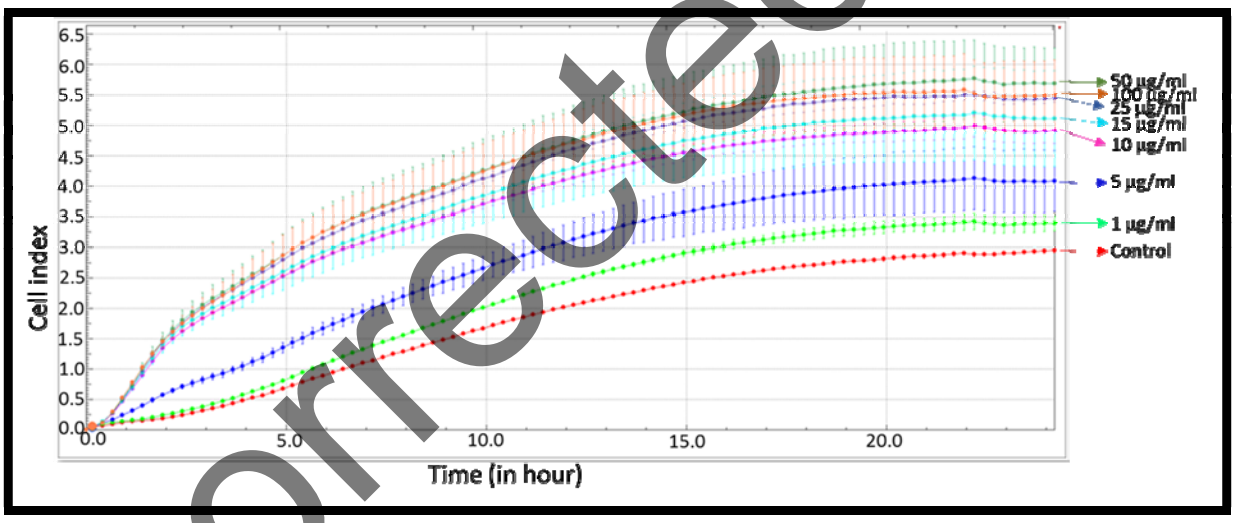

Figure 3. The binding ability of PEO-1 cells to fibronectin by RTCA. PEO-1 cells were allowed to incubate for 24 h on 16-well plates coated with 1-50 µg/ml fibronectin concentrations. The control wells were uncoated with fibronectin. As mentioned in section of Materials and Methods, the impedance was monitored at 15 min interval during 24 h.

Effects of Calcium on PEO-1 Cell Binding

Figure 4 depicts that the involvement of Ca^{2+} increases in the mechanism of cell adhesion to fibronectin. PEO-1 cells were plated onto 16-well plates of which were previously coated with 50 µg/ml fibronectin in the presence or absence of various concentrations of TN, TG or EGTA. The results demonstrate that integrin-mediated

PEO-1 adhesion to fibronectin is reduced by Ca^{2+} mobilisation from both extracellular Ca^{2+} and intracellular Ca^{2+} pool. The addition of all Ca^{2+} mobilisation compounds resulted in a decrease in CI back down to zero at 24 h time point because of the cells rounding and detaching from the well bottom.

Figure 4A demonstrate the change in cell index occurred within the first few hours of exposing PEO-1 cells to the all concentrations of TN. The rate of adhesion of PEO-1 cells to fibronectin decreased as a dose-dependent manner. 0.5 and 1 µg/ml of TN indicated no effects on PEO-1 adhesion ability as compared to the control cells. The proportion of adherent cells reduced to a minimal level by the presence of 20 µg/ml TN. The results suggest that extracellular calcium mobilisation reduced the adhesion of high metastatic PEO-1 cells to fibronectin and might induce anoikis of PEO-1 cells that is remaining unclear.

Addition of all concentrations of TG reduced the cell adhesion to fibronectin upon time-dependent manner as compared to the control cells (CI value is 0.4) (Figure 4B). After 24 h incubation time, even the addition of lowest concentration (50 nM) of TG decreased the attachment of PEO-1 cells to fibronection to zero. The results suggest the adhesion of PEO-1 cells to fibronectin decayed by the inhibition of endoplasmic reticulum pump via TG treatment.

Treatment of PEO-1 cells with various concentrations of EGTA was also reduced the rate of adhesion within 5 h and then remain unchanged (Figure 4C).

The Role of PKB/AKT Signal Pathway on PEO-1 Cell Binding to Fibronectin

FPA-124 was used for inhibiting PKB/AKT (Protein kinase B) signal pathway. Impact of inhibition of PKB/AKT on cell binding was tested for different concentration (5 µM, 15 µM, 25 µM, 35 µM) of FPA 124 (Figure 5). Percentage of the impact of different FPA-124 concentrations on cell binding at 24 h in PEO-1 is given in Figure 5B.

Figure 5A demonstrates the attachment of the control cells (without FPA-124). Figure 5B shows that the cells treated with 5 µM inhibitor increased gradually (CI: 1.9 and CI: 1.4 after 24 h incubation time) (Figure 5B). Increasing the concentration of inhibitor about three

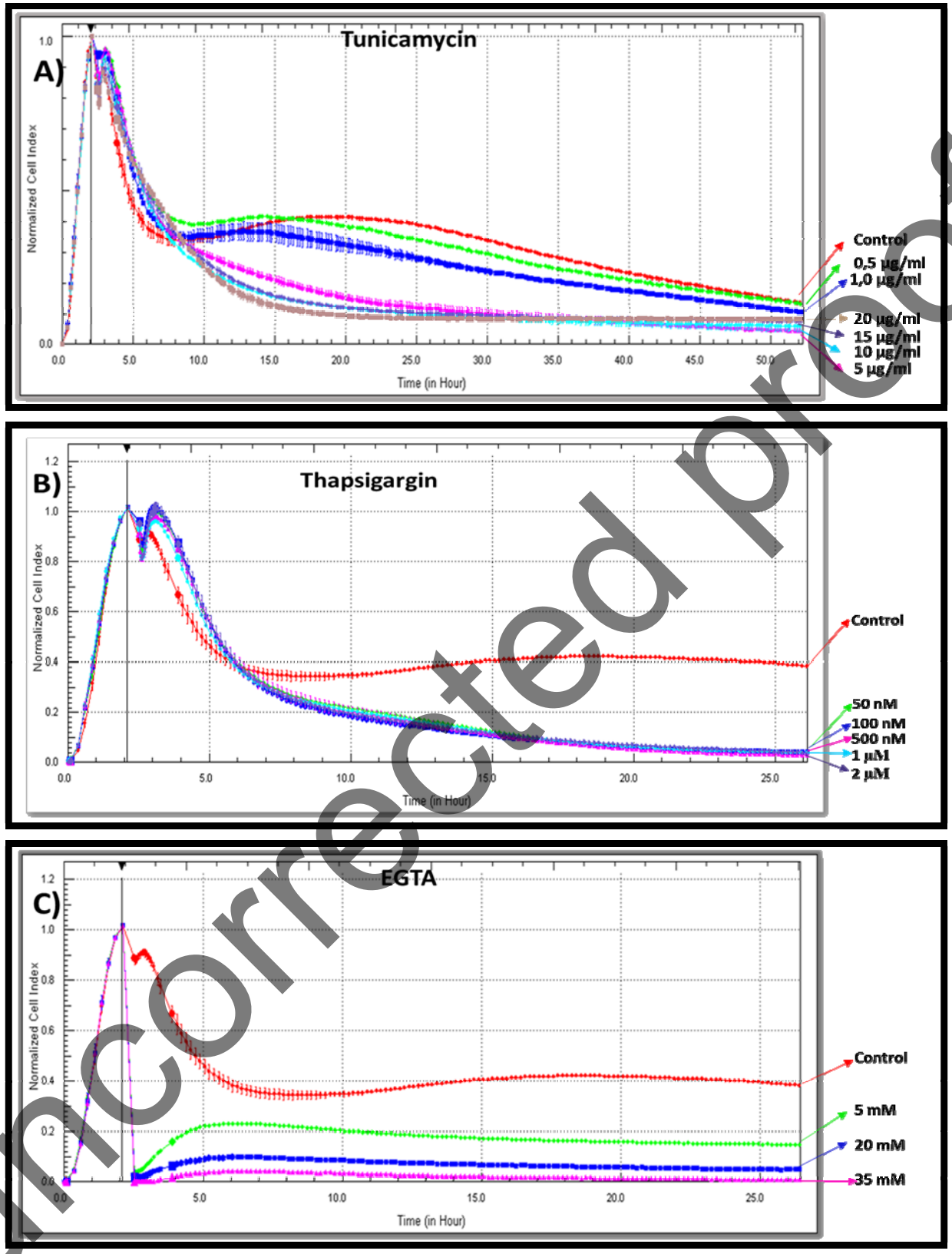

Figure 4. Effects of TN, TG and EGTA on PEO-1 binding to 50 µg/ml fibronectin after 24 h. **A)** PEO-1 cells were treated with 0,5-1-5-10-20 µg/ml of TN; **B)** with 50-100-500 nM-1-2 µM TG and **C)** with 5-20-35 mM of EGTA for 24 h after plated onto 50 µg/ml fibronectin. The rate of cell adhesion was calculated according to NCI formula.

times showed the different effects on PEO-1 cells attachment to fibronectin. For the first few hours, the cell attachment to fibronectin raised after adding 15 µM FPA inhibitor, however longer incubation time reduced the binding of PEO-1 cells to the background level (CI: 0.3). The results here suggest that FPA inhibitor might cause rapid focal contacts between cell-fibronectin in a dose dependent manner. Higher concentrations of FPA-124 (25 and 35 µM) inhibited the binding of cells to fibronectin immediately after adding into the culture medium.

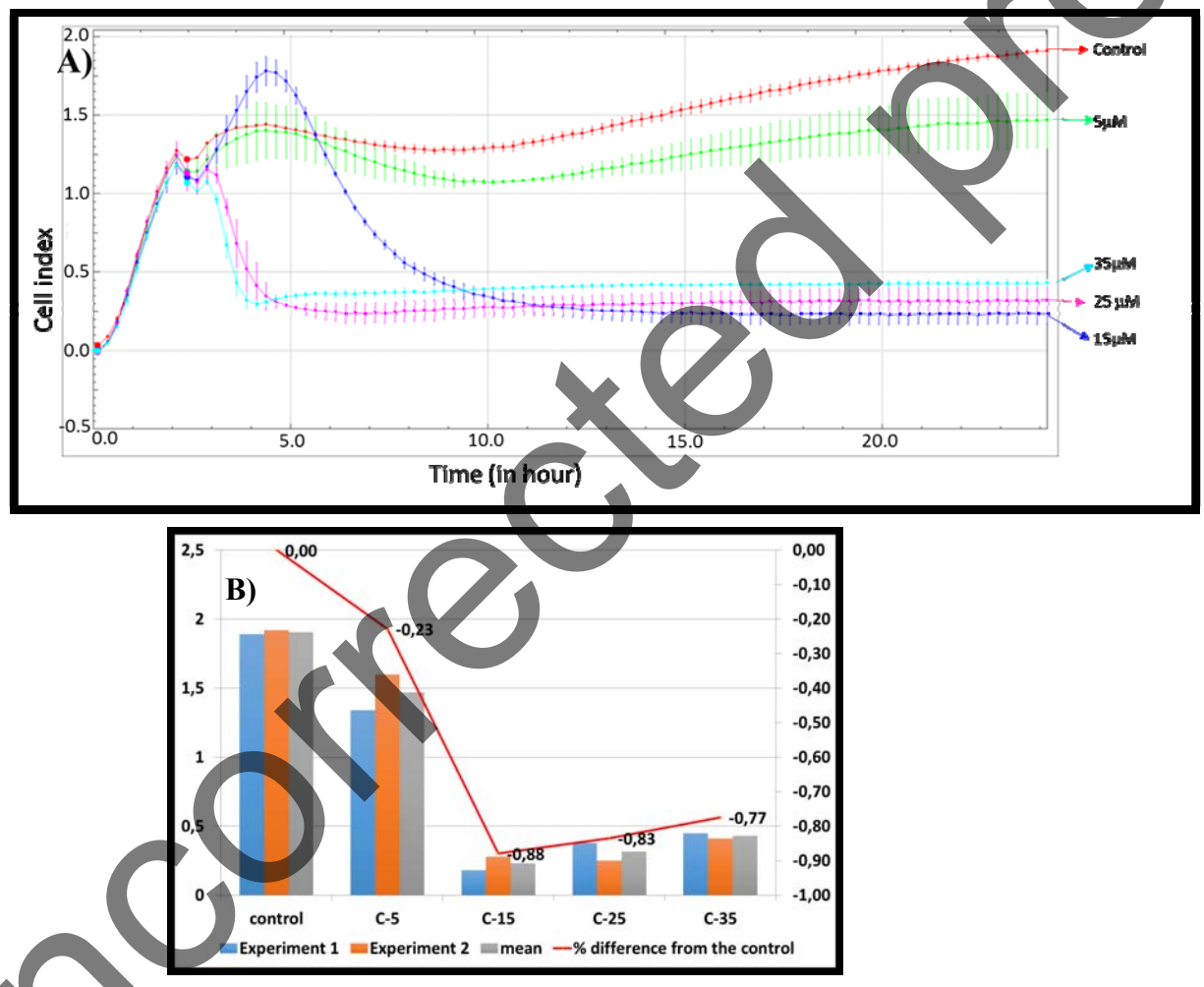

Figure 5. The role of PKB/AKT molecules on PEO-1 cells binding to fibronectin **(A)** and the impact of different FPA-124 concentrations on cell binding at 24 h **(B)**. 16-wells plates were prepared as mentioned in section of Materials and Methods. Various concentrations (5-35 µM) of FPA-124 inhibitor were added to each well. Inhibitor was not added to the control groups. **(B)** Left axis is the cell index at 24 h, right axis is the percentage differences from the control group.

The Role of Cardamonin on PEO-1 Cell Binding to Fibronectin

The effects of cardamonin on PEO-1 cell binding to fibronectin were investigated by RTCA system and adding various concentrations (3.13, 6.25, 12.5, 25, 50 and 100 µM) of cardamonin. The results showed in Figure 6A and B. Addition of 100 µM cardamonin to the cells inhibited the cell binding to fibronectin about 64 %.. Except for, 3.13 µM treatment of cardamonin, all other concentrations of cardamonin inhibited the cell binding to fibronectin as compared to the control cells plated on uncoated wells. Treatment of cells with lowest concentration (3.13) of cardamonin stimulated the rate of cell binding to fibronectin about 13 % as compared to control cells. The results showed that the degradation of β-catenin in the cytoplasm by cardamonin has an important role in PEO-1 cell binding to fibronectin.

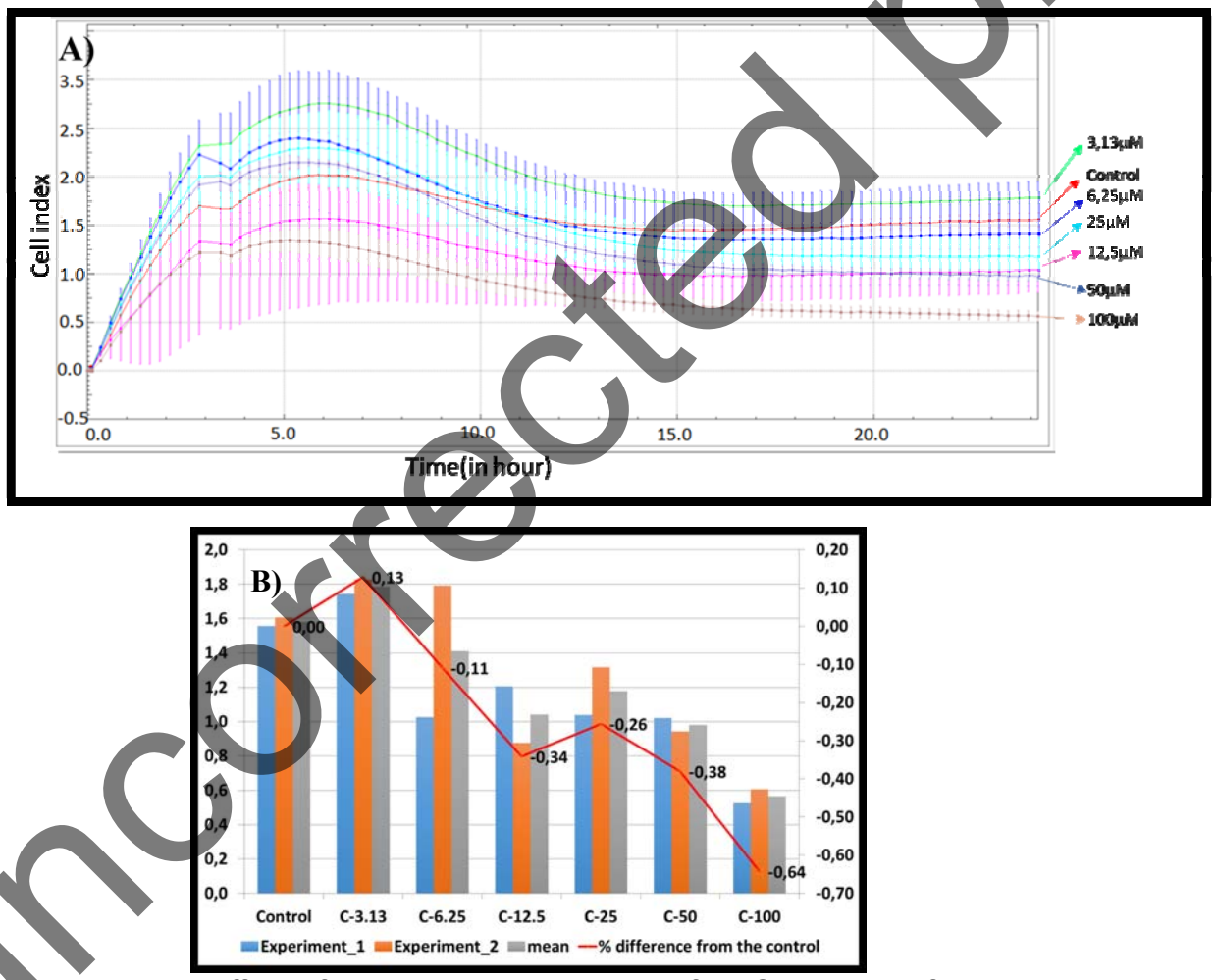

Figure 6. The effect of cardamonin on binding of PEO-1 cells to fibronectin **(A)** and percentage of the impact of different cardamonin concentrations on PEO-1 cell binding to fibronectin at 24 h **(B)**. Wells were coated with 50 µg/ml fibronectin at +4 °C overnight. The cells were transferred into each well and then incubated with cardamonin (concentration range between 3.13 µM and 100 µM) for 24 h. The results represented are the means of two wells. **(B)** Left axis is the cell index at 24 h, right axis is the percentage differences from the control group.

Discussion

The adhesion and integrin activation are not only important to metastasize the ovarian cancer cells onto the peritoneal mesothelial surface of the abdominal cavity (17), but also in the resistance to anoikis of the EOC (epithelial ovarian cancer) spheroids. It has been reported that some binding proteins might be involved in the implantation of ovarian cancer metastasis (18). Especially, the CD44 molecule, which is a receptor for ECM protein hyaluronic acid, plays a crucial role in mediating mesothelial binding of ovarian cancer cells and β1 integrins such as α5β1 integrin (a receptor for fibronectin), α3β1 (a receptor for fibronectin, collagen and laminin) and αv (a receptor for fibronectin and vitronectin) expressed by ovarian cancer cells, mediate ovarian cancer cell binding to peritoneal mesothelium (18). We found in our experiment that the expression level of αv is two folds larger than the control group in PEO-1 cells. The other integrin expression levels of α4, β1 and β6 subunits are higher about 32 %, 48 %, 84 % than the control group respectively. The αvβ1 fibronectin receptor most likely contributes to integrin-mediated binding of PEO-1 cells. These results are consistent with the previous findings. Carduner et al., (19) reported that αv has an important role in ovarian cancer progression. Lessan et al., (20) informed that β1 integrin especially may responsible for adhesion of ovarian cancer cells.

Fibronectin has been detected in ovarian cancer cell metastases and is also present in ascites (21-22). Fibronectin contributes to the formation, adhesion and disaggregation of ovarian cancer cell spheroids (23). Therefore, the investigation of the binding capacity of ovarian cancer cells to fibronectin should be useful for targeted therapies. Binding studies here suggested that PEO-1 cells could be able to adhere to fibronectin in a dose dependent-manner and maximum binding rate was reached after adhesion of cells to 50 µg/ml fibronectin. The binding capacity of PEO-1 cells was also measured in the presence or absence of calcium. Because the integrin-mediated cell adhesion is regulated by extracellular Ca^{2+} ions (24) and increased calcium concentration in cytoplasm is an essential of various signal transduction pathways such as proliferation and apoptosis and thus is also crucial in cancer (25). TN induces the endoplasmic stress by inhibiting glycosylation and lead to accumulation of unfolded proteins. Endoplasmic stress has a critical role in

regulating cell death (26). Our results clearly indicated that increased calcium levels in cytoplasm of PEO-1 cells by either extracellular or intracellular stores is essential for binding of these cells to fibronectin. Intracellular calcium increasing in PEO-1 cells reduced the cell adhesiveness and also might cause the programmed cell death induced upon cell detachment from ECM.

PKB/Akt is a serine/threonine kinase that has an important role in cell survival, cell proliferation, invasion and adhesion to ECM in various types of cells such as HUVECs (27), breast cancer cells (28) and lung adenocarcinoma (29). The overexpression of PI3K/Akt related genes is observed in ovarian cancer tissues and (30). Moreover, fibronectin adhesion promotes the metastatic behaviour upon the type of cancer cells through the FAK–PI3K/Akt pathway (31). Xing et al., (32) suggest that Akt activation promoted by fibronectin might have a critical role in cell survival. In here, inhibiton of PKB/Akt by FPA inhibitor decreased the rate of adhesion of PEO-1 cells to fibronectin in a dose dependent manner, suggesting that PKB/Akt participates in the signaling of adhesion in these cells.

In this study, we found that the inhibition of β-catenin by cardamonin reduced PEO-1 cells binding to fibronectin. β-catenin is activated downstream signal molecule from Akt and Akt-mediated phosphorylation of β-catenin results in its accumulation in the cytosol and the translocation into nucleus, thus upregulates genes related to cell proliferation such as c-Myc, cyclin D1 and D2 (33). The function of β-catenin may be necessary for the adhesive and signalling responses required for cancer. The inhibition of β-catenin activity causes suppression of several cancer hallmarks and that might be as useful as a putative drug target (34). Pramanik et al.,(35) found that inhibition of β-catenin signalling blocked pancreatic tumour growth. Verma et al., (36) showed that small interfering RNA's (siRNA) directed against β-catenin reduced β-catenin dependent gene expression and growth of colon cancer cells. In this study, we found that the binding ability of PEO-1 ovarian cells to fibronectin decreased by the treatment of 100 μM cardamonin suggesting that β-catenin might involve in adhesion process of PEO-1 cells to fibronectin as well as Akt. This process could be mediated by integrin especially αv subunit, but further experiments are required for investigating the role of αv subunit under this circumstances.

Conclusion

In conclusion, PEO-1 cells binding to fibronectin via integrins could be related to intracellular Ca^{+2} mobilisation and Akt singaling. However, the sensitivity of these cells against to anoikis is still remained to be investigated.

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Declaration of interest

The authors declare that there is no conflicting financial interest.

References

1. Manso AM, Kang SM, Ross RS. Integrins, Focal Adhesions and Cardiac Fibroblasts. J Investig Med. 2009; 57(8): 856–860.
2. Aoudjit F and Vuori K. Integrin Signalling in Cancer Cell Survival and Chemoresistance. Chemotherapy Res and Pract. 2012; 2012: 1-16.
3. Hirohashi S and Kanai Y. Cell adhesion system and human cancer morphogenesis. Cancer Sci. 2003; 94: 575-581.
4. Van den Hoogen C, van der Horst G, Cheung H, Buijs JT, Pelger RC, van der Pluijm G. Integrin αv expression is required for the acquisition of a metastatic stem/progenitor cell phenotype in human prostate cancer. Am J Pathol. 2011; 179:2559–2568.
5. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. Biochimica et Biophysica Acta-Molecular Cell Research. 2013; 1833: 3481-3498.
6. Lu JG, Li Y, Li L, Kan X. Overexpression of osteopontin and integrin αv in laryngeal and hypopharyngeal carcinomas associated with differentiation and metastasis. J Cancer Res Clin Oncol. 2011; 137:1613–1618.
7. Puiffe ML, Le Page C, Filali-Mouhim A, Zietarska M, Ouellet V, Tonin PN, Chevrette M, Provencher DM, Mes-Masson AM. Characterization of ovarian cancer ascites on cell invasion, proliferation, spheroid formation, and gene expression in an in vitro model of epithelial ovarian cancer. Neoplasia 2007; 9: 820–829.

8. Kenny HA, Kaur S, Coussens LM, Lengyel E. The initial steps of ovarian cancer
cell metastasis are mediated by MMP-2 cleavage of vitronectin and fibronectin, J. Clin. Invest. 2008; 118: 1367–1379
9. Bodnar L, Stanczak A, Cierniak S, Smoter M, Cichowicz M, Kozlowski W, Szczylik C, Wieczorek M, Lamparska-Przybysz M. Wnt/β-catenin pathway as a potential prognostic and predictive marker in patients with advanced ovarian cancer. J of Ovarian Research. 2014; 7: 16-26.
10. Hur J and Jeong S. Multitasking β-catenin: From adhesion and transcription to RNA regulation. Animal Cell and Systems. 2013; 17: 299-305.
11. Uchino M, Kojima H., Wada K. Nuclear beta-catenin and CD44 upregulation characterize invasive cell populations in non-aggressive MCF-7 breast cancer cell. BMC Cancer. 2010; 10: 414-417.
12. Chau WK, Ip CK, Mak AS. C-Kit mediates chemoresistance and tumor-initiating capacity of ovarian cancer cells through activation of Wnt/beta-catenin-ATP-binding cassette G2 signaling. Oncogene. 2012; 10: 103-107.
13.
14. Tran NL, Adams DG, Vaillancourt RR, Heimark RL. Signal transduction from N-cadherin increases Bcl-2. Regulation of the phosphatidylinositol 3-kinase/Akt pathway by homophilic adhesion and actin cytoskeletal organization. J Biol Chem. 2002; 277: 32905-32914.
15. Sharma M, Chuang WW, Sun Z. Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3beta inhibition and nuclear beta-catenin accumulation. J Biol Chem. 2002; 277: 30935-30941.
16. Rahim S and Üren A. A real time electrical impedance based technique to measure invasion of endothelial cell monolayer by cancer cells. J. Vis. Exp. 2011; 50: 2792-2797.
17. ZhangJD.2016.https://www.bioconductor.org/packages/devel/bioc/vignettes/RTCA/inst/doc/aboutRTCA.pdf
18. Sawada K, Mitra AK, Radjabi AR, Bhaskar V, Kistner EO. Tretiakova M, Jagadeeswaran S, Montag A, Becker A, Kenny HA. Loss of E-cadherin promotes ovarian cancer metastasis via α 5-integrin, which is a therapeutic target. Cancer Res. 2008; 68: 2329–2339.
19. Strobel T, Cannistra S. B1-Integrins partly mediated binding of ovarian cancer cells to peritoneal mesothelium *in vitro*. Gynecologic Oncology 1999;73: 362-367.
20. Carduner L, Leroy-Dudal J, Picot CR, Gallet O, Carreiras F, Kellouche S. Ascites-induced shift along epithelial-mesenchymal spectrum in ovarian cancer cells: enhancement of their invasive behaviour partly dependant on av integrins. Clinical and Experimental Metastasis. 2014; 31: 675-688.
21. Lessan K, Aguiar DJ, Oegema T, Siebenson L, Skubitz APN. CD44 and β1 integrin mediate ovarian carcinoma cell adhesion to peritoneal mesothelial cells. American J of Pathology 1999; 154: 1525-1537.
22.
23. Bignotti E, et al. Gene expression profile of ovarian serous papillary carcinomas: identification of metastasis-associated genes. Am J Obstet Gynecol. 2007; 196: 245.e1–11
24. Wilhelm O, Hafter R, Coppenrath E, Pflanz M, Schmitt M, Graeff H. Fibrin-fibronectin compounds in human ovarian tumor ascites and their possible relation to the tumor stroma. Cancer Res. 1988; 48(12):3507–3514.

25. Mitra AK, Sawada K, Tiwari P, Mui K, Gwin K, Lengyel E. Ligand-independent activation of c-Met by fibronectin and α5β1-integrin regulates ovarian cancer invasion and metastasis. Oncogene 2011; 30:1566–1576.
26. Tsaktanis T, Kremling H, Pavsic M, von Stackelberg R, Mack B, Fukumori A, Steiner H, Vielmuth F, Spindler V, Huang Z, Jakubowski J, StoeckleinNH, Luxenburger E, Lauber K, Lenarcic B, Gires O. Cleavage and cell adhesion properties of human epithelial cell adhesion molecule (HEPCAM). The Journal of Biological Chemistry 2015; 290: 24574-24591.
27. Giorgi C, Bonova M, Pinton P. Inside the tumor: p53 modulates calcium homeostasis. Cell Cycle 2015; 147: 933-934.
28. Nami B, Donmez H, Kocak N. Tunicamycin-induced endoplasmic reticulum stress reduces in vitro subpopulation and invasion of CD44+/CD24- phenotype breast cancer stem cells. Exp Toxicol Pathol. 2016; 68(7): 418-426.
29. Hwang H-J, Chung H S, Jung T W, Ryu J Y, Hong H C, Seo J A, Kim S G, Kim N H, Choi K M, Choi D S, Baik S H, Yoo H J. The dipeptidyl peptidase-IV inhibitor inhibits the expression of vascular adhesion molecules and inflammatory cytokines in HUVECs via Akt- and AMPK-dependent mechanisms. Molecular and Cellular Endocrinology 2015; 405: 25-34.
30. Leung H-W, Wang Z, Yue G-L G, Zhao S-M, Lee K-M J, Fung K-P, Leung P-C, Lau B-S C, Tan N-Hua. Cyclopeptide RA-V inhibits cell adhesion and invasion in both estrogen receptor positive and negative breast cancer cells via PI3K/AKT and NF-kB signalling pathways. Biochimica et Biophysica Acta-Molecular Cell Research. 2015; 1853: 1827-1840.
31. Piegeler T, Schlapfer M, Dulli RO, Schwartz DE, Borgeat A, Minshall RD, Beck-Schimmer B. Clinically relevant concentrations of lidocaine and ropivacaine inhibit TNFa-induced invasion of lung adenocarcinoma cells in vitro by blocking the activation of Akt and focal adhesion kinase. British Journal of Anaesthesia 2015; 115: 784-791.
32. Gwak H, Kim S, Dhanasekaran DN, Song YS. Resveratrol triggers ER stress-mediated apoptosis by disrupting N-linked glycosylation of proteins in ovarian cancer cells. Cancer Letters. 2016; 371: 347-353.
33. Yousif NG. Fibronectin promotes migration and invasion of ovarian cancer cells through up-regulation of FAK–PI3K/Akt pathway. Cell Biol Int. 2013; 38 (2014): 85–91.
34. Xing H, Weng D, Chen G, Tao W, Zhu T, Yang X, Meng L, Wang S, Lu Y, Ma D. Activation of fibronectin/PI-3K/Akt2 leads to chemoresistance to docetaxel by regulating survivin protein expression in ovarian and breast cancer cells. Cancer Letters 2008; 261: 108–119.
35. Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, Mills GB, Kobayashi R, Hunter T, Lu Z. Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. Journal Biological Chemistry. 2007; 282: 11221-11229.
36. Luu HH, Zhang R, Haydon RC. Wnt/beta-catenin signalling pathway as a novel cancer drug target. Curr Cancer Drug Targets. 2004; 4: 653-671.

37. Pramanik KC, Fofaria NM, Gupta P, Ranjan A, Kim SH, Srivastava SK Inhibition of β-catenin signaling suppresses pancreatic tumor growth by disrupting nuclear β-catenin/TCF-1 complex: critical role of STAT-3. Oncotarget. 2015;10;6(13):11561-74.

38. Verma UN, Surabhi RM, Schmaltieg A, Becerra C and Gaynor RB. Small Interfering RNAs Directed against β-Catenin Inhibit the in Vitro and in Vivo Growth of Colon Cancer Cells.Clinical Cancer Research. 2003;9(4): 1291-1300.