

RESEARCH ARTICLE

Dexamethasone Disrupts Cytoskeleton Organization and Migration of T47D Human Breast Cancer Cells by Modulating the AKT/mTOR/RhoA Pathway

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Abstract

Background: Glucocorticoids are commonly co-administered with chemotherapy to prevent drug-induced allergic reactions, nausea, and vomiting, and have anti-tumor functions clinically; however, the distinct effects of GC on subtypes of tumor cells, especially in breast cancer cells, are still not well understood. In this study, we aimed to clarify the effect of GC on subtypes of T47D breast cancer cells by focusing on apoptosis, cell organization and migration, and underlying molecular mechanisms. **Materials and Methods:** The cell scratch test was performed to observe the cell migration rate in T47D cells treated with dexamethasone (Dex). Hoechst and MTT assays were conducted to detect cell survival and rhodamine-labeled phalloidin staining to observe cytoskeleton dynamics. Related factors in the AKT/mTOR pathway were determined by Western blotting. **Results:** Dex treatment could effectively inhibit T47D breast cancer cell migration with disruption of the cytoskeletal dynamic organization. Moreover, the effect of Dex on cell migration and cytoskeleton may be mediated by AKT/mTOR/RhoA pathway. Although Dex inhibited T47D cell migration, it alone may not induce cell apoptosis in T47D cells. **Conclusions:** Dex in T47D human breast cancer cells could effectively inhibit cell migration by disrupting the cytoskeletal dynamic organization, which may be mediated by the AKT/mTOR/RhoA pathway. Our work suggests that glucocorticoid/Dex clinical use may prove helpful for the treatment of breast cancer metastasis.

Keywords: Dexamethasone - T47D breast cancer cells - cell migration - cytoskeleton

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Introduction

Breast cancer is a clinically terrible disease occurring in the mammary gland epithelial, which is of high risks in the women (Cuzick et al., 2011; Prat et al., 2011). The breast cancer could perform invasion and metastasis to escape from the primary tumor and penetrating into the blood circulation, further endanger people's lives (Turnbull et al., 2008). So far, many of the risk factors for breast cancer have been well established. These include, age, race, a family history of breast cancer, genetic susceptibility, benign breast disease, early age at menarche, late age at menopause, and nulliparity (Armstrong et al., 2000; Graf et al., 2010). Based on comprehensive gene expression profiling, breast tumors are classified into at least three major subtypes, luminal, human epidermal growth factor receptor2⁺ (HER2⁺), and basal like (Polyak, 2011). Each of these subtypes has different risk factors for incidence, response to treatment, risk of disease progression, and preferential organ sites of metastasis.

Nowadays, the effective treatments of breast cancer are based on surgery, radiotherapies, chemotherapies, hormonal therapies and adjuvant treatments (Gianni et

al., 2010; Siegel et al., 2012). Although numerous agents are in various phases of clinical development, however, few of these shows promise for treating all breast cancers. Since the intra- and inter-tumor heterogeneities provide great challenges for developing successful therapies (Savarese et al., 1993), it still needs to identify novel targeted signaling pathways and reagents that regulate malignant progression of breast cancers, which would be therapeutically important.

In recent years, glucocorticoid (GC) has been implicated in modulating invasion and metastasis of multiple tumors (Yamamoto et al., 2002; Frankfurt et al., 2004; Banciu et al., 2008). GC is a type of steroid hormones secreted by adrenal cortex or synthesized, and commonly co-administered with chemotherapy to prevent drug-induced allergic reaction, nausea, and vomiting. It has been reported that GC may affect growth and chemosensitivity of carcinoma cells via diverse mechanisms. Dexamethasone (Dex) is a synthetic GC, which is often used in clinic to treat all kinds of diseases (Kostaras et al., 2014; Sau et al., 2014), such as breast cancers. For example, Nakayama et al found that combination of palonosetron and Dex was an antiemetic

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treatment of choice for patients with breast cancer treated with anthracycline (Nakayama et al., 2013). Treated patients with breast cancer liver metastases using hepatic arterial infusion of Dex, and found that it could significantly improve the median survival (Ang et al., 2013). These studies establish that Dex would help to the clinical treatment of breast cancers. However, a recent study shows that Dex could also enhance metastasis and tumorigenicity in MDA-MB-231 cells, a TNBC cell line, while decreased tumorigenicity in two non-TNBC cell lines, MCF7 and T47D cells (Alain et al., 2012; Silva et al., 2012). Therefore, it needs further clarification that the role of Dex in the treatment of breast cancers, especially its distinct effect on subtypes of breast cancers.

To verify the role of Dex in different subtypes of breast cancers, we focus on the human mammary breast cancer cell line T47D. T47D cells, established from pleural effusion of a human breast cancer patient, have been reported to have steroid hormone receptors to estrogen, progesterone, glucocorticoid, and androgen (Ono et al., 1987). It has been reported that Dex may decrease the tumorigenicity in T47D cells, as well as inducing its calcitonin receptor (CTR) expressions (Kurokawa et al., 1991). But it still not clear whether Dex affects other tumorous characterizations of T47D cells. Here we demonstrate that migratory activity of T47D cells was inhibited by Dex treatment, without increased cell apoptosis. Dex has been showed to dramatically inhibit cytoskeletal reorganizations, which may be responsible for the decreased cell migrations. Moreover, the effect of Dex on cytoskeletal dynamics may be mediated by AKT/mTOR/RhoA signaling. Thus, our work proposed a promising target of treatment strategies for breast cancers, and demonstrates that Dex may work as a coadjuvant of tumor metastasis inhibitor in treatment of breast cancers, at least in part of non-TNBC.

Materials and Methods

Cell culture

The T47D human breast cancer cell line was purchased from American Type Culture Collection (ATCC, USA). All the cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 100IU/ml penicillin and 100 µg/mL streptomycin (GIBCO, USA), cultured in 5% CO₂, 37°C incubator. The cells were passaged with 0.25% trypsin when they grew to 90% density.

Cell scratch assay

The spreading and migration capabilities of T47D cells were assessed using a scratch wound assay which measures the expansion of a cell population on surfaces (Fronza et al., 2009). The cells were seeded into 12-well tissue culture dishes containing pre-coated coverslips. A linear wound was generated in the monolayer with a sterile 100µl plastic pipette tip. Any cellular debris was removed by washing the coverslips with phosphate buffer saline (PBS). Then the cells were treated with 0µm, 1µm, 10µm and 100µm dexamethasone (Sigma, USA) for 48h, and no additives were used as internal controls. Finally, the cells were fixed with 4% paraformaldehyde for 15min

and observed under microscope and measured with Image pro plus software. Three representative images from each coverslip of the scratched areas under each condition were photographed to estimate the relative migration cells for quantifications.

Cell survival assay

The T47D cells were treated with 0µm, 1µm, 10µm and 100µm Dex for 24 and 48h, and then prepared for Hoechst staining for cell death detections and MTT assay for cell viability detections. Briefly, 1.0×10⁵ cells/mL T47D cells were plated in 6-well plates. After pharmacological manipulations, cells were directly stained with Hoechst kit from Beyotime (Jiangsu, China). The cell counting was carried out through the use of National Institutes of Health software ImageJ. As for MTT assays, cells were plated at a density of 1×10⁴ cells/well in 96-well plates for 12 h prior to pharmacological manipulations. In total, 20µl MTT (5mg/ml in PBS, Sigma, USA) was added to each well 4 h prior to the desired endpoint to dissolve the formazan crystals. The absorbance was measured at 570nm in a 96-well plate reader. The percentages of cell viability were calculated according to OD value.

Immunofluorescence staining

To detect the cytoskeletal dynamics in Dex treated T47D cells, the Rhodamine Phalloidin R415 probes were applied to stain the F-actin fibers. For the preparation of Rhodamine Phalloidin staining, T47D cells were plated with 1.0×10⁵ cells/mL in 12-well plates. After pharmacological manipulations (Dex 100µm), cells were fixed with 4% Paraformaldehyde (PFA) and 4% sucrose in PBS for 30min and then permeabilized with 0.25% Triton-X 100 in PBS for another 5min at room temperature. After washing extensively, Rhodamine Phalloidin R415 probes were added into these cells. Then after a second washing for three times, the cover slips were mounted onto glass slides with antifade reagent with DAPI for nuclear labeling.

Western blot

The proteins were extracted from cells treated with 100 µM Dex by lysis buffer (PBS+1% Triton X-100) at 0, 3, 12 and 24h, and protein concentrations were measured with BCA Protein Assay kit (Thermo Fisher Scientific, USA). Then the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF, Bio-rad, USA) membranes following the standard procedures. Next, the membranes were blocked by 5% non-fat dry milk in PBST (PBS with 0.1% Tween 20, pH7.6), then incubated with appropriate primary antibodies and appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz, USA), and developed with ECL Plus luminescent reagents (GE Healthcare, USA). Anti-AKT, anti-p-AKT, anti-mTOR, anti-p-mTOR antibodies were purchased from Cell Signaling Technology (Danvers, USA); and anti-RhoA, anti-β-actin antibodies were purchased from Millipore (Billerica, USA).

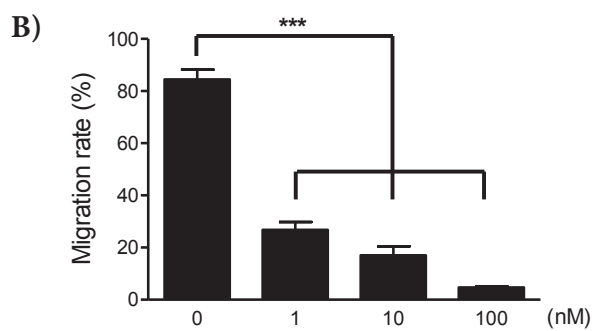
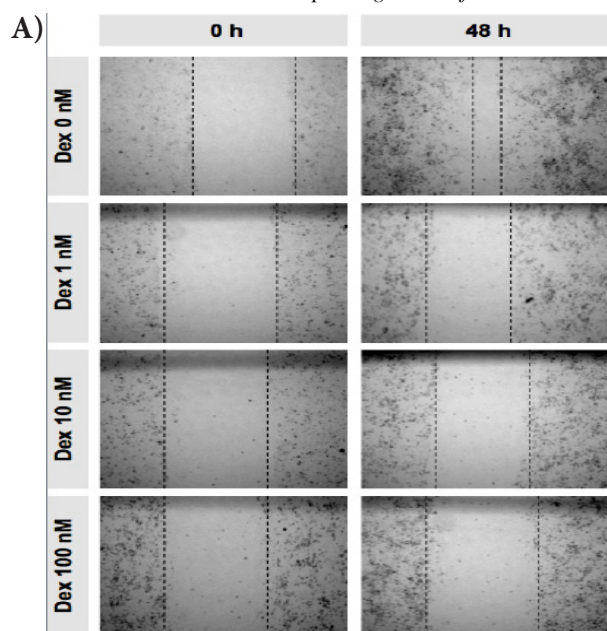


Figure 1. Dex Inhibits Cell Migration in T47D Human Breast Cancer Cells

Statistical analysis

All data were expressed as mean standard deviation (s) and analyzed by SPSS software. The comparisons between two groups were used student t test, a $p < 0.05$ was considered as statistically significant.

Results

Dex inhibits cell migration in T47D human breast cancer cells

Dex has showed distinct effects on different subtypes of tumor cells, especially in breast cancer cells (Lu et al., 2005; Lu et al., 2006a; 2006b). For example, Dex does not affect the proliferation rate of MDA-MB-231 breast cancer cells (a triple negative breast cancer cell line, TNBC), but enhances their metastasis and tumorigenicity (Alain et al., 2012). However, treatment of Dex decreases tumorigenicity in two non-TNBC cell lines, MCF7 and T47D cells (Silva et al., 2012). Moreover, Dex could also induce calcitonin receptor (CTR) expressions in T47D cells (Kurokawa et al., 1991). Therefore, how Dex decreases the tumorigenicity in T47D cells and whether other tumorous characterizations are altered by Dex treatment are still to be verified. To detect the effects of Dex on T47D cells, we examined the spreading and migration capabilities of T47D cells by Dex treatment. The cell scratch results showed that the migration rate

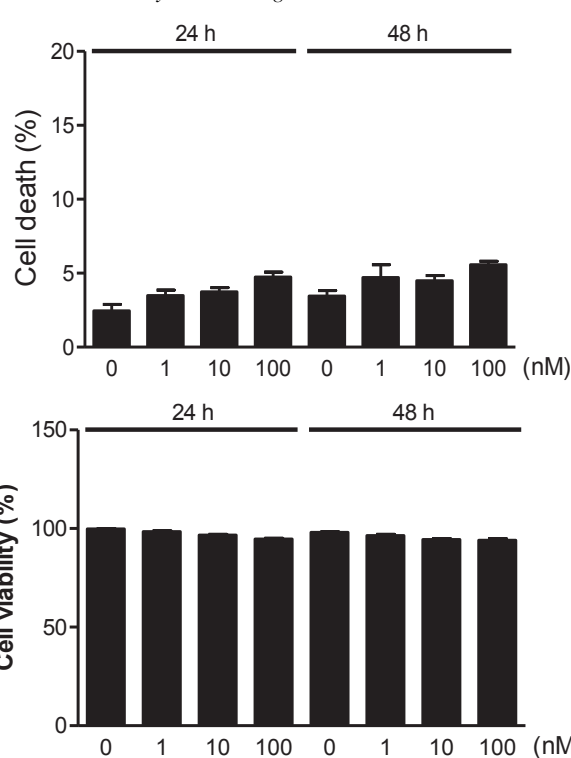


Figure 2. Dex Induces Minimal Apoptosis in T47D Human Breast Cancer Cells

was inhibited by Dex treatment in a dose-dependent manner (Figure 1A and B). In details, after 48h recovering, T47D cell migrates into the wound, and the remainder wound is only about 15.4% of the beginning. However, the rapid wound healing was dramatically inhibited by Dex treatment. With Dex treatment, the migration rate is reduced to 26.8% (1 μ M Dex), 17.0% (10 μ M Dex) and 4.7% (100 μ M Dex), compared to the 84.5% (control). All these results suggested that Dex treatment may reduce migration viability in T47D human breast cancer cells.

Dex induces minimal apoptosis in T47D human breast cancer cells

Dex has been showed to induce cell apoptosis in many malignancy tumor cells, but some preceding experiment indicated that Dex may not affect cell survival in some subtypes of breast cancer cells (Silva et al., 2012). Therefore, to further investigate whether Dex will affect T47D cell viability under the condition of inhibited migration, we detected the cell survival by Hoechst staining and MTT assays. The T47D cells were treated with Dex by 1 μ M, 10 μ M and 100 μ M for 24 and 48h. Then these cells were collected for the subsequent examinations. The results showed that Dex may slightly increase apoptotic cell numbers, such as 4.7% (100 μ M Dex for 24h) and 5.6% (100 μ M Dex for 48h) compared to 2.5% (controls). But these differences were not significant ($p > 0.05$, Figure 2A). Again, the results of MTT assays for cell viability were consistent with the apoptotic results, showing that the cell viability was not significantly altered by Dex treatment (Figure 2B). These results are partly consistent with previous reports, suggesting that the effect of Dex on cell apoptosis may be distinct by the subtypes of cells. Taken together, our work demonstrates that Dex

may not affect the cell survival in T47D breast cancer cells, although it could inhibit the cell migration.

Dex dramatically impairs the cytoskeletal dynamics in T47D human breast cancer cells

To explore the mechanism of how Dex inhibits cell migration in T47D cells, we focused on the cytoskeletal dynamics. Because Dex has been reported to inhibit tendon cell migration that is correlated with decreased gene expression of α -SM actin (Tsai et al., 2003), it is of high possibility that Dex may affect the cytoskeletal dynamics in T47D cells. Thus, we carried out F-actin staining by Rhodamine-labeled Phalloidin probes in T47D cells. Rhodamine-phalloidin is a high-affinity F-actin probe conjugated to the red-orange fluorescent dye and selectively stains F-actin. T47D cells were pre-treated by Dex for 0 to 24h, and then visualized by the fluorescence microscope. Red fluorescence indicated that as elapsed Dex treatment, F-actin fibers were dramatically reduced in a time- and dose-dependent manner, while DAPI signals labeled blue indicated the nuclear locations (Figure 3). Thus, our results show that Dex treatment may impair the cytoskeleton dynamics and organizations in T47D cells, which may be responsible for the blocked cell migrations.

Dex impairs cytoskeletal dynamics via AKT/mTOR/RhoA pathways in T47D human breast cancer cells

To investigate the molecular mechanism of how Dex disrupts the cytoskeletal dynamics and blocks cell migration in T47D cells, we focused on the well-known AKT/mTOR pathways. AKT/mTOR pathway is one of the major signaling pathways that have been identified

as important in the tumor development (Altomare et al., 2004; Jozwiak et al., 2010; Atif et al., 2014). It has been shown that AKT/mTOR pathways regulate tumor cell migration and cancer metastasis (Yothaisong et al., 2013; Xing et al., 2014). For example, inactivation of AKT/mTOR activity suppresses F-actin reorganization and migration in various tumor cell lines, and this effect may be mediated by downregulation of RhoA protein expression and activity (Liu et al., 2010). Thus, we examined the protein levels of AKT/mTOR/RhoA pathways. The results showed that the expression of phosphorylated AKT (*p*-AKT) and phosphorylated mTOR (*p*-mTOR), which were indicators of AKT and mTOR activity, are reduced as prolonged Dex treatment for 24h. Also, RhoA, a small GTPase protein known to regulate the cytoskeletal dynamics, was inactivated as the downstream target of AKT/mTOR pathway treated by Dex (Figure 4). These results were consistent with previous works on cytoskeleton studies and supported the notion that Dex may impair cytoskeletal dynamics through AKT/mTOR/RhoA pathway in T47D cells.

Discussion

Cell proliferation, adhesion, migration and invasion were closely related to cancer progression, and cell migration was one of the key steps of cancer invasion and metastasis (Pal et al., 2013; Perlikos et al., 2013). Therefore, inhibition of tumor cell migration might be important method to prevent tumor progression. In this

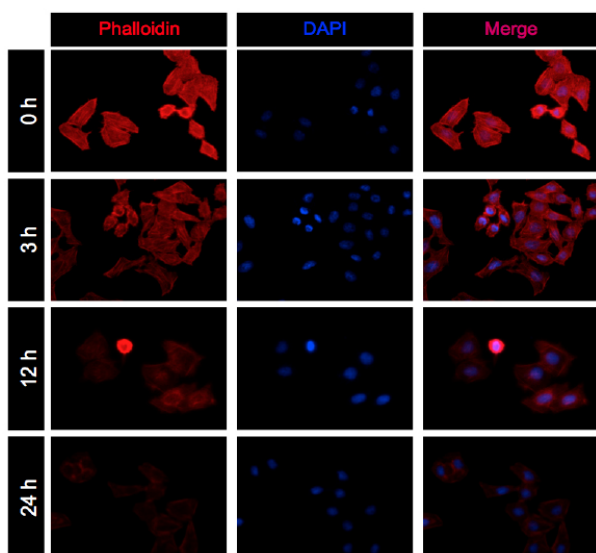


Figure 3. Dex Dramatically Impairs the Cytoskeletal Dynamics in T47D Human Breast Cancer Cells

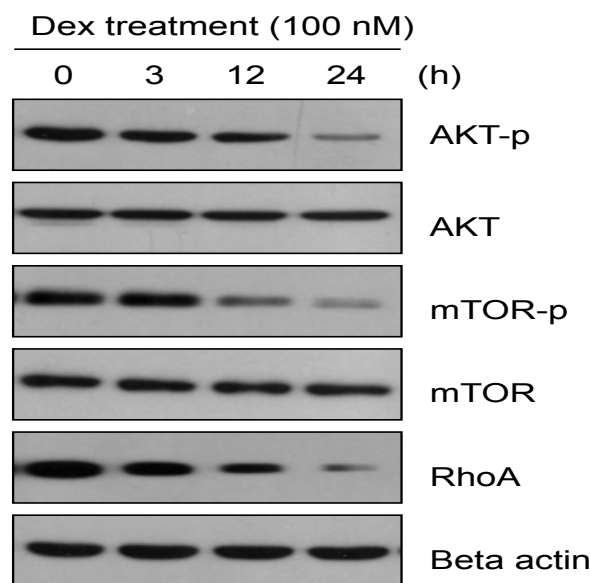


Figure 4. Dex Impairs Cytoskeletal Dynamics Via AKT/mTOR/RhoA Pathways in T47D Human Breast Cancer Cells

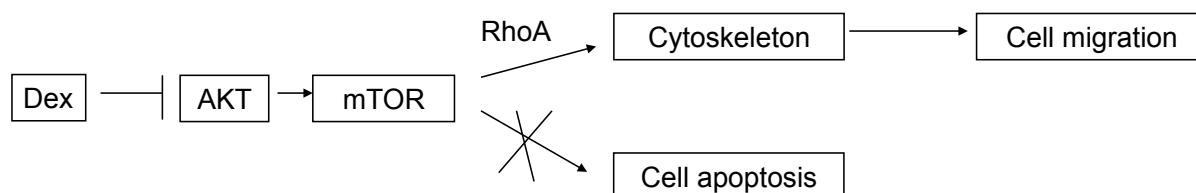


Figure 5. Schematic Representation Highlighting the Mechanism of Dex’s Effect on Cytoskeleton and Migration in T47D Human Breast Cancer Cells

study, we demonstrate that dexamethasone (Dex), a coadjuvant medicine for patients receiving chemotherapy, could effectively inhibit T47D breast cancer cell migration by disrupting the cytoskeletal dynamic organizations. Moreover, the effect of Dex on cell migration and cytoskeleton may be mediated by AKT/mTOR/RhoA pathway. Intriguingly, although Dex exhibits superb effects of inhibiting T47D cell migrations, it alone may not induce cell apoptosis in T47D cells, suggesting that Dex is better to work with other chemotherapeutics to clinically treat breast cancers (Figure 5).

It has long been appreciated that glucocorticoid/Dex is commonly co-administered with chemotherapy to prevent drug-induced allergic reaction, nausea, and vomiting. However, the alone effect of Dex on tumor cells is different in various types of cells. For example, treatment of Dex resulted in early axillary lymph node metastases, and increased the number of lung and liver metastases in an orthotopic xenograft model of a triple negative breast cancer cell line (MDA-MB-231) in nude mice (Alain et al., 2012). While Dex could decrease tumorigenicity in two non-TNBC cell lines, MCF7 and T47D cells (Silva et al., 2012). Moreover, Dex has been reported to induce apoptosis of osteocytes, as well as inducing autophagy for self-protections (Xia et al., 2010). Recently, many reports have demonstrated that Dex is widely used in clinical cancer therapy, besides its role in decreasing tumorigenicity. For example, prophylactic Dex could decrease the frequency and severity of grade 3 skin rash in cancer therapy (Wu et al., 2013). Moreover, combined use of Dex with 5-HT3 antagonist, and NK1 receptor inhibitor may be helpful for the patients suffering from chemotherapy-induced nausea and vomiting (Kumagai et al., 2014). Therefore, it is necessary to determine the molecular mechanism of how Dex affect tumor cell survival, metabolism and development. Here, to reveal the role of Dex in breast cancers, especially to distinct effect on different subtypes of breast tumor cells, we investigate the role of Dex on cell organizations and migrations of T47D human breast cancer cells. Our results at least clarify that although Dex could not induce dramatic T47D cell apoptosis, it could disrupt the cytoskeletal organizations and migrations via AKT/mTOR/RhoA axis. These results afford reasons to take glucocorticoid/Dex as coadjuvant medicine for breast cancer therapies.

The AKT/mTOR pathway is a master intracellular signaling pathway which is important in apoptosis and hence cancer, e.g. breast cancers (Paplomata et al., 2014), non-small-cell lung cancers (Alvarez et al., 2007) and colorectal cancers (Pandurangan, 2013). Therefore, targeted drugs of AKT/mTOR pathway might be helpful for treatment of cancers, especially leading to cell apoptosis (Lou et al., 2014; Sui et al., 2014; Wang et al., 2014). Interestingly, in current studies, we found that AKT/mTOR pathway was inactivated by Dex treatment, without dramatic cell apoptosis. Instead, the Dex-mediated inactivation of AKT/mTOR pathway tends to block the cytoskeleton organizations and cell migrations. The compartmental effects of AKT/mTOR pathway on tumor inhibitions afford us novel insights into the pharmacological designs of targeted drugs. Again,

RhoA was regarded as a prominent regulatory factor in the regulation of cytoskeletal dynamics, transcription, cell cycle progression and cell transformation (Hou et al., 2013). Schmidt et al found that the expression of RhoA was notably higher in malignant prostate cancer cells compared to benign prostate cells (Schmidt et al., 2012). In our study, the expression of RhoA was inactivated when the cell migration was inhibited by Dex treatment, enhancing the notion that Dex could indeed disrupt the cytoskeleton organizations and cell migrations at least in T47D human breast cancer cells.

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