Stretching Induces Overexpression of RhoA and Rac1 GTPases in Breast Cancer Cells

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Rho GTPases are well known for regulating cell morphology and intracellular interactions. They can either be oncogenic or tumor suppressors. However, these proteins are associated with the acquisition of malignant features by cancer cells. It has been reported that the overexpression of protein markers of Rho family members such as RhoA and Rac1 is linked with carcinogenesis and the progression of a variety of human tumors. In this paper, the expression of RhoA and Rac1 activity in various types of breast cancers cell lines is evaluated. These cells are preconditioned by mechanically stretching them to simulate the extracellular physical forces placed upon on cancer cells. It is observed that stretching the cancer cells induces significantly higher expression of RhoA and Rac1 markers when compared to non-stretched cells and stretched control cells in vitro. This stretching strategy helps to detect and quantify the signal when it is too weak to be detected. Furthermore, stretching enhances the assay by leading to overexpression of markers and makes the assay more sensitive. It is hypothesized that this inexpensive and relatively sensitive assay can potentially aid in the development of a diagnostic tool for cancer screening.

1. Introduction

Various signaling pathways are highly involved in breast malignancies. The cancer cells receive and process signals either via direct cellular contact or indirectly via receptors. These signals regulate normal cellular processes such as protein synthesis, cell differentiation, and growth, or apoptosis.[1] Mutation and the consequential alteration of these signaling processes are considered the fundamental basis of cancer. However, due to the complex nature of these signaling pathways, it is always a challenge to study them. For example, different receptors such as estrogen and progesterone have an established role in undifferentiated breast cancer. While the activation of PI3K/AKT and JUN/MAPK pathways may suppress breast cancer development. Interaction between these pathways is again a factor to consider. It is known that PI3K/AKT pathway is targeted in estrogen positive breast cancer for therapeutic applications as this pathway is activated by the 17 beta-estradiol.[2] Furthermore, MAP kinase receptors cross-talk with estrogen receptors to enhance the estrogen mediated signaling.[3] These pathways receive signals from extracellular signals via the cellular receptors and are responsible for the intracellular and cellular changes. It is now widely accepted that extracellular physical factors also play a significant role in cellular undifferentiation and mutation of malignant cells. The Rho-GTPase signaling pathway regulates cellular morphology and intracellular interaction, therefore, attracted interest in cancer biology research.[4]

Rho GTPases belong to a family of regulatory small guanosine triphosphate (GTP) binding proteins in the Ras superfamily. They are small molecules of 21–25 kDa that share structural homology and when activated bind to GTP. The studies carried out in the early 1990s indicated that the Rho proteins regulate cell morphology through actin cytoskeleton.[5] However, recent studies show that they also influence gene expression, cell proliferation, and survival[6]; all cellular functions that play a crucial role in carcinogenesis.[7]

Rho GTPases are responsible for actin cytoskeleton rearrangement after the extracellular matrix (ECM) mechanical properties variations. Mammals have 22 Rho GTPases and most of them affect the cell morphology. The primary mechanism of the Rho GTPases action is the change in the membrane-associated actin cytoskeleton. The widely investigated members of this family are RhoA, Rac1, and Cdc42.[8] RhoA, the first member of this family, is activated by mechanical stress, ROCK, or mammalian Diaphanous.[9] Rac1, another member of the family, is also activated by mechanical stimuli and affects cyclic stretching-induced cells and stress fibers reorientation. However, different Rho-Guanine nucleotide exchange factors and Rho-GTPase-activating proteins are regulated during mechanotransduction. These Rho-GEFs and Rho-GAPs are actively involved in actin cytoskeletal remodeling in mecha-responses.[10]

Though reported as both oncogenic and tumor suppressors, RHO GTPases are associated with the acquisition of malignant
features by cancer cells\textsuperscript{[11]} The Rho GTPases also play a key role in pathological processes such as cancer progression, inflammation, and wound repair.\textsuperscript{[46,12]} RhoA and Rac1 as members of Rho GTPases family are involved in the regulation of different cellular processes including microfilament organization and cell–cell contact. They also perform essential functions in actin cytoskeleton organization. Rho A helps in the formation of stress fibers and focal adhesions of cells. Rac1 contributes to lamellipodium formation and membrane ruffling.\textsuperscript{[14]} In cancer, the Rho family GTPases influence many processes such as cell-transformation, survival, invasion, metastasis, and angiogenesis. Overexpression of RhoA and Rac1 have been linked with carcinogenesis and progression of various human malignancies.\textsuperscript{[7,11b,13,14]}

RhoA and Rac1 have been described as an oncogene due to its overexpression within malignant tumors in vivo.\textsuperscript{[46,15]} Moreover, RHO signaling has been implicated in tumor invasion and metastasis through the exertion of dual processes of cell contraction and protrusion.\textsuperscript{[16]} Rac1 has also been reported to possess similar roles as other GTPases including promoting cancer initiation and metastasis. However, Rac1 is also highly involved in mediating cell motility and invasion, which are key features in metastatic cells.\textsuperscript{[17]}

The RhoGTPases are highly involved with the ECM and there are reports stating that RhoGTPases are involved in the synthesis of ECM via mechanical signal transduction.\textsuperscript{[18]} Also, RhoGTPases are involved in many other cellular processes such as actin dynamics, cell motility, cell adhesion, cell proliferation, and apoptosis. In cancer, the Rho GTPases are known to influence many processes such as cell-transformation, survival, invasion, metastasis, and angiogenesis. Overexpression of RhoA and Rac1 have been linked with carcinogenesis and progression of various human malignancies.\textsuperscript{[7,11b,13,14]}

2. Experimental Section

2.1. Mechanical Cell-Stretching Device

The electromagnetic actuation device and procedure had been previously described by Yadav et al.\textsuperscript{[20]} Briefly, the platform consists of four primary components: i) a PDMS device with embedded permanent magnets (PMs), ii) a holding clip of the static strain condition, iii) a mounting stage with electromagnets for cyclic strain conditions, and iv) a deformable 200-µm thick membrane. First, the PDMS device was cleaned with isopropanol and de-ionized (DI) water. The device was subsequently treated with oxygen plasma, bonded with the deformable membrane, and cured for 1 h at 80 °C. In a typical experiment, cyclic stretching was achieved via a linear stage driven by a computer-controlled system. Cells are plated on the deformable PDMS membrane and incubated, as explained in Section 2.2.

2.2. Cell Maintenance and Co-Culture in the Stretching Device

All the breast cancer cell lines (MDA-MB-231, SKBR3, MCF-7) and control human mammary fibroblasts (HMF) were obtained from the American Type of Culture Collection (ATCC). The cells were grown and maintained in DMEM/F12 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultured in T75 flasks at 37 °C in a humified atmosphere with 5% CO\textsubscript{2}. Before adding the cells, the device was sterilized with 80% ethanol and washed three times with sterile 1X Hank’s balanced salt solution (HBSS). Ultraviolet (UV) irradiation was applied for 30 min, 400 µL of DMEM-F12 media was then added to the cell-stretching device and incubated for 1 h to further enhance biocompatibility. For seeding, 80% confluent cells were harvested and counted with a hemocytometer, a total number of 75 000 cells were seeded into the device. The device was then incubated for 24 h to achieve the optimal adherence and growth of the cells on the membrane. After incubation, the cells were then washed three times with HBSS, and 400 µL of media was added. The mechanical strain was then applied, as described in Section 2.3.

2.3. Application of Strain on the Cells

The device was placed on the cell-stretching platform and kept at 37 °C to maintain a physiological temperature in a 5% CO\textsubscript{2} environment. Cyclic stretching was applied to the cells with 1.4% strain at 0.01 Hz and 50% duty cycle as optimized in the previous studies.\textsuperscript{[20]} Strecthing applied over two different time points, that is, 2 h and 4 h, following preparation for ELISA analysis (Section 2.4).

2.4. Chemical Cell Lysis

After stretching, the cells were washed and trypsinized, pelleted and counted before the cells were lysed. The cells were subjected to 1% NP-40, 20 mM EDTA, 50 mM Tris-HCl pH 7.5 for chemical lysis. After 30 min incubation on ice, the lysate was centrifuged at 5000 RPM for 5 min to pellet cell debris. The supernatant was collected and stored at −20 °C for the ELISA as follows.

2.5. Measurement of RhoA and Rac1 by Enzyme-Linked Immunosorbent Assay (ELISA)

2.5.1. RhoA

Levels of RhoA were determined using the Ras Homolog Gene Family, Member A (RhoA), ELISA Kit from MyBioSource,
Inc. San Diego, California, USA. The samples were prepared according to the manufacturer’s instructions. Briefly, cells were lysed as explained in Section 2.4. The cells were then added (100 µL per well) to 96 well pre-coated plates and incubated for 1 h at 37 °C followed by 100 µL of detection reagent A and incubated for 1 h at 37 °C. After the subsequent washing, 100 µL of detection reagent B was added and incubated for 30 min at 37 °C. After washing, 90 µL of horseradish peroxidase (HRP) substrate solution was added, and the plates were further incubated for 20 min. The enzyme-substrate reaction was terminated by the addition of stop solution and the color change was measured using a microplate reader (SpectraMax) at a wavelength of 450 nm. The concentration of RhoA in the samples were determined by comparing the O.D of the samples to the standard curve.

2.5.2. Rac1

Levels of Rac1 were determined using the Ras Related C3 Botulinum Toxin Substrate 1 (Rac1), ELISA Kit (MyBioSource, Inc. San Diego, California, USA). The samples were prepared according to the manufacturer’s instructions. Briefly, the cells were lysed using the cell lysis buffer as explained in Section 2.4, and then added (100 µL per well) to 96 well pre-coated plates and incubated for 1 h at 37 °C followed by 100 µL of detection reagent A and incubated for 1 h. After the subsequent washing, 100 µL of detection reagent B was added and incubated for 30 min. After washing, 90 µL of HRP substrate solution was added and the plates were incubated for a further 20 min. The reaction was stopped by adding 50 µL of stop solution. Optical density was determined using a microplate reader (SpectraMax) at 450 nm. The concentration of Rac1 in the samples were determined by comparing the O.D of the samples to the standard curve.

2.6. Statistical Analysis

Statistical analysis of all experiments was performed using Prism 7 software (GraphPad Software, San Diego, CA). All data were shown as the mean ± standard error of the mean for three technical replicates. The concentration of RhoA and Rac1 was normalized against the cell count.

3. Results and Discussion

Three breast cancer (MDA-MB-231, SKBR3, and MCF-7) and control (HMF) cell lines were subjected to mechanical stretch and lysed as per the workflow illustrated in Figure 1. Briefly, the cells were plated on PDMS deformable membrane and were kept in an incubator under normal cell culture conditions (37 °C, 5% CO₂, 95% humidity). After 24 h of incubation, the membrane was stretched by applying cyclic strain. After stretching the cells were washed, trypsinized, and counted. Figure 2a shows the number of cells seeded was 75,000, however, only 10,000 survived after 4 h of stretching due to the apoptosis of cells.20 Figure 2b shows the representative fluorescence image of MDA-MB-231 with the cell morphology and distribution before stretching. Figure 2c,d show the images after 2 h stretching and 4 h stretching, respectively. Although the cells look confluent, the total number of cells recovered were low. As explained in our previous paper,20 there was apoptosis after 4 h, however, the loss of cells in 2 h is not statistically significant. The cells respond to external stress transmission and reorganize their cytoskeleton architecture by reconstructing the actin stress fibers over time. Also, the filopodia had been observed in this stage which shows that the cells are active.

3.1. Protein Biomarkers Recovery from Cells

All the viable cells during the experiment were harvested for RhoA and Rac1 quantification. These kinases are intracellular and hence require membrane disruption for releasing them into the sample for quantification. Cell lysis or cell disruption is a method where the outer boundary of the cell membrane is broken down or destroyed to release the intercellular content. The mild surfactant NP-40 has an ability to disrupt the cells by hydrophobic–hydrophilic interactions. It is a popular buffer for studying proteins that are cytoplasmic or membrane-bound. The cells were lysed by using NP-40 as explained in Section 2.4. The supernatant with protein markers was collected for further quantification by ELISA.

3.2. Estimation of RhoA and Rac1

The levels of the released marker, RhoA and Rac1, were detected by using sandwich ELISA. The expression level of protein markers was measured in both breast cancer and control cells.

3.3. Quantification of RhoA by ELISA

Protein expression of RhoA was observed in both breast cancer cells and control cells (Figure 3a). However, the expression of RhoA in tumor sample was higher than control sample. As shown in Figure 3a, cancer (MDA-MB-231) cell shows 0.042 pg mL⁻¹ of RhoA expression while non-cancer sample (HMF) shows 0.024 pg mL⁻¹. The difference in expression level of RhoA in cancer and non-cancer supports the fact that RhoA is overexpressed in breast malignancies. However, it was interesting to note that there was no statistically significant difference in the expression of RhoA between tumor and control cells. Overexpression of RhoA protein had been reported in many malignancies such as breast, lung, colon, and bladder cancers.11b,22 Also, the protein expression level is greater in more advanced stages than those more benign.

3.4. Quantification of Rac1

The expression of Rac1 displayed a positive correlation as seen with RhoA. As shown in Figure 3c, Rac1 expression of breast cancer cells (MDA-MB-231) is 0.036 pg mL⁻¹ and control cell
Non-statistical difference between the expression of both RhoA and Rac1 and between breast-cancer cells and control cells implies that the expression of these RhoGTPases is cancer related. It has been reported that the expression of these GTPases is highly regulated. The overexpression of these proteins will occur in cancerous cells,[23] but most probably overexpresses during the stress on the cancer cells due to the involvement of the Rho pathway.[24] RhoGTPase signaling appears to be a response mechanism to the forces exerted on the cells by the extracellular matrix.[10a] Hence, it is important to attempt to replicate physical forces on the cells and quantifying the expression differences in the Rho proteins.

3.5. Quantification of RhoA and Rac1 After the Application of Cyclic Strain

To determine whether the expression of Rho proteins is affected by cyclic stretching in both breast-cancer and control cells, we examined the relative expression level of RhoA and Rac1 before and after stretch. We stretched the MDA-MB-231 and HMF cells for 2 h, lysed the cells, and quantified using ELISA. As shown in Figure 4a, we observed that the relative expression of RhoA before stretching was 0.042 pg mL$^{-1}$, while after stretching was 0.109 pg mL$^{-1}$ in MDA-MB-231 cells. In HMF cells, there was RhoA 0.024 pg mL$^{-1}$ before and 0.050 pg mL$^{-1}$ after stretch. Next, we investigated Rac1 expression before and after stretching (Figure 4b). We observed a similar trend in Rac1 expression in MDA-MB-231, where 0.036 pg mL$^{-1}$ was observed before stretching that increased to 0.075 pg mL$^{-1}$ after stretching. In HMF cells, there was Rac1 0.023 pg mL$^{-1}$ before and 0.036 pg mL$^{-1}$ after stretch. The results suggested that MDA-MB-231 cells stretched for the prolonged period increased the expression of these markers. As shown in Figure 4c the expression of the same proteins was not reciprocated in control cells. This simultaneous increase of RhoGTPase proteins in both cancer and non-control cells made it difficult to differentiate. Although we have previously reported that strain forces placed upon cells affect their morphology with a difference more prominent in cancer cells,[20] this difference was not clearly reflected in these biomarkers. Low level of expressed biomarkers also makes it more difficult to quantify these markers and increases error rates to observe a statistically significant difference.

Figure 1. Schematic representation of the assay for the analysis of RhoA and Rac1 protein markers. The breast cancer cells and control cells were seeded in the PDMS device. After 24 h of incubation, the device was placed on the cell stretching platform and cyclic mechanical strain was applied. The cells were trypsinized and chemically lysed for the release of RhoA and Rac1 markers. The levels of markers were determined by using ELISA. Further, the markers were quantified before and after stretching.
3.6. Time-Dependent Activation of RhoA and Rac1

As we observed, differences in protein expression between non-stretched and 2 h stretched MDA-MB-231 cells, we increased the stretching time to 4 h. As shown in Figure 5a, we observed the same trend of RhoA expression in the three breast cancer cell lines. There is an increased level of the marker in first 2 h of stretching and interestingly there is sharp rise after 4 h of subsequent stretching. In the same way as shown in Figure 5b, the Rac1 level is increased in first 2 h of mechanical strain and sharp increase after prolonged stretch.

3.7. Detection of Protein Markers in Multiple Cell Lines

We extended our assay with two further types of breast cancer cells. We used SKBR3 and MCF7, compared to normal human mammary fibroblast. These cells were chosen, as they are the most common cell lines used in breast cancer studies and offer variety in a cancer type. MDA-MB-231 cells are isolated from invasive ductal carcinoma and are both estrogen and progesterone receptor negative. MCF-7 cells, on the other hand, are positive for estrogen, progesterone, and glucocorticoid receptors and retain properties of mammary epithelium. SKBR3 cells carry similar properties like MDA-MB-231 cells and just differ in being sensitive to All-trans retinoic acid. All the cells were stretched in similar ways. As expected, the RhoA results revealed that the signals were much higher in all the cancer samples after prolonged stretching, that is, 4 h stretch time (Figure 5c). We observed that the relative expression of RhoA before stretching was 0.042 pg mL$^{-1}$, while after stretching was 0.276 pg mL$^{-1}$ in MDA-MB-231 cells. In SKBR3 cells, there was RhoA 0.026 pg mL$^{-1}$ before and 0.159 pg mL$^{-1}$ after stretch. Similarly, in MCF7 cells, there was RhoA 0.026 pg mL$^{-1}$ before and 0.237 pg mL$^{-1}$ after stretch. In the control (HMF) cell, the RhoA was 0.024 pg mL$^{-1}$ before and 0.075 pg mL$^{-1}$ after stretch. At the same time, as shown in Figure 5d, Rac1 results also correlated with the previous one. We observed that the relative expression of Rac1 before stretching was 0.036 pg mL$^{-1}$, while after stretching was 0.266 pg mL$^{-1}$ in MDA-MB-231 cells. In SKBR3 cells, there was Rac1 0.035 pg mL$^{-1}$ before and 0.248 pg mL$^{-1}$ after stretch. In the control (HMF) cell, the Rac1 was 0.024 pg mL$^{-1}$ before and 0.075 pg mL$^{-1}$ after stretch. Also, in control (HMF) cell, the Rac1 was 0.023 pg mL$^{-1}$ to 0.058 pg mL$^{-1}$. It is also important to remark that we could easily discriminate the signals between cancer and control.
Figure 3. ELISA detection of Rho proteins in breast cancer and control cells. a) The relative expression levels of RhoA in breast cancer (MDA-MB-231) and control (HMF) sample. b) The standard curve of the RhoA. c) The relative expression levels of Rac1 in cancer (MDA-MB-231) and control (HMF) sample. d) The standard curve of the Rac1. Standard significance was determined by pairwise comparisons between two conditions using a Student's t-test. Error bar represents the standard deviation of the experiments (n = 3).

Figure 4. ELISA detection of RHO markers before and after stretching in MDA-MB-231 and HMF cells. a) The relative expression level of RhoA in breast cancer cell before and after stretching. b) The relative expression of Rac1 in breast cancer cell before and after stretching. c) The relative expression level of RhoA in control cell before and after stretching. d) The relative expression of Rac1 in control cell before and after stretching. Standard significance was determined by pairwise comparisons between two conditions using Student's t-test. *p ≤ 0.05; **p ≤ 0.005, ***p ≤ 0.0005. Error bar represents the standard deviation of the experiments (n = 3).
samples after prolong stretching. It is clearly visible that there were not any significant changes after 4 h of stretching in control sample for both the cases. The significant difference in the expression of these markers is due to the activation of the RhoGTPase pathway in cancer. It was interesting to note that both RhoA and Rac1 show very similar trends. This result is in accord with various reports showing that RhoA and Rac1 regulate each other to modulate different cell processes.

4. Conclusion

In conclusion, we demonstrated that RhoA and Rac1 protein markers were expressed more significantly in breast cancer cells compared to control cells. When stretched for a prolonged period, these breast cancer cells significantly increased the overexpression of the same markers when compared to the control cells. It is well known that the overexpression of RhoA and Rac1 is linked with carcinogenesis and progression of numerous human tumors. The RhoA protein marker has been reported in a number of malignancies such as breast,[25] lung, gastric,[26] colon cancers, while Rac1 has been shown to increase in breast[27] cancer. Rho GTPases are now considered a potential therapeutic target for cancer. Our stretching strategy could be used for amplifying protein signaling, thus making the assay more sensitive. This would afford a relatively simple, sensitive, and inexpensive method to detect cancer protein biomarkers. Most importantly, the stretching of malignant cells enhances the precision of the assay by leading to overexpression of markers and makes the assay more sensitive. We believe that our assay could find potential application in detecting the protein markers by amplifying the signal via stretching. This hypothesis offers advantages of amplifying the signal of the markers when the signal is too weak to detect. We envisage that our hypothesis is not limited to breast cancer, but could be widely applied for other human malignancies.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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