Video Article

Characterization of Cell Membrane Extensions and Studying Their Roles in Cancer Cell Adhesion Dynamics

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Abstract

The cell membrane's extension repertoire modulates various malignant behaviors of cancer cells, including their adhesive and migratory potentials. The ability to accurately classify and quantify cell extensions and measure the effect on a cell's adhesive capacity is critical to determining how cell-signaling events impact cancer cell behavior and aggressiveness. Here, we describe the *in vitro* design and use of a cell extension quantification method in conjunction with an adhesion capacity assay in an established *in vitro* model for adrenocortical carcinoma (ACC). Specifically, we test the effects of DKK3, a putative tumor suppressor and a pro-differentiation factor, on the membrane extension phenotype of the ACC cell line, SW-13. We propose these assays to provide relatively simple, reliable, and easily interpretable metrics to measures these characteristics under various experimental conditions.

Video Link

The video component of this article can be found at https://www.jove.com/video/56560/

Introduction

Dysregulated WNT signaling plays a critical role in adrenocortical malignancies[1]. The methods used in this study investigate whether silencing of DKK3, a negative regulator of WNT signaling, represents a dedifferentiation event in the adrenal cortex and promotes tumor formation in the context of cell-extension repertoire changes. DKK3 is a 38 kDa secreted glycoprotein with an N-terminal signal peptide and previous studies have demonstrated that its enforced expression resulted in cell cycle arrest, inhibited aggressive malignant behavior, and reversed epithelial-mesenchymal transition[2].

The malignant behavior of adrenocortical carcinoma (ACC) and other cancers is, in part, influenced by the ability of tumor cells to interface with the surrounding surfaces, including the extracellular matrix, which in turn facilitates tumor cell invasion and migration[3]. The role of specific cell membrane extensions in cancer progression is being increasingly demonstrated in various contexts, primarily via the formation of filopodia. For example, overexpression of L-type calcium channels has been found to induce filopodia formation and promote tumor cell invasion[4]. Similarly, Fascin, an actin binding protein minimally expressed in normal tissue, is also overexpressed in cancer cells in association with filopodia formation[5]. Lobopodia formation enables non-malignant fibroblasts to migrate effectively through the extra-cellular matrix, however, it has been shown that fibrosarcoma cells rely on metalloproteinase activity in lieu of lobopodia to facilitate cell migration and invasion[6]. We have shown that tumor suppressors, including Ras association domain family 1 isoform A (RASSF1A) and DKK3, can function to alter cytoskeletal elements and promote lamellipodia formation and stymie invasive properties[7,8].

As such, it is critical to characterize the effects of genes involved in carcinogenesis and their relationship to cell-membrane extension alterations, specifically assessing filopodia, lobopodia, and lamellipodia formation under test conditions. Current state-of-the art techniques include the use of increasingly sophisticated microscopy methods, fluorescent labeling, and/or complex computer algorithms for data acquisition and interpretation. While these methods provide new and powerful analytic tools, their complexity limits their widespread use and adaptability in cell biology experiments. Furthermore, the precise quantification and observation of changes in cell extension morphology is not typically measured[9,10]. In contrast, we introduce a technique here that accurately quantifies cell extension alterations using standard microscopic techniques and readily adaptable *in vitro* methods. These methods also quantify each cell extension type simultaneously for each cell analyzed and determine overall changes in the cell membrane extension repertoire. We also show how these changes can relate to cell adhesion properties.

As an experimental example, we will use a previously created cell line of SW-13, designated SW-DDK3, which has been stably transfected with pCMV6-Entry/DKK3 plasmid vectors and constitutively overexpresses DKK3, a differentiating factor in the adrenal gland. Non-transfected SW-13 cells and SW-13 cells stably transfected with empty vector (pCMV6-Entry), designated SW-Neo, will serve as experimental controls.

Protocol

1. Cell Extension Characteristics

1. Maintain SW-13, SW-Neo, and SW-DKK3 cells in a standard humidified incubator at 37.0 °C and 5% CO_2 in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 10,000 U/mL penicillin and streptomycin (designated as 'growth medium').
    1. Count cells using a hemocytometer, and plate 5,000 cells per well for SW-13, SW-Neo, and SW-DKK3 lines into separate 6-well plates and grow overnight in growth medium on sterile glass coverslips.

2. After overnight growth, for each well, aspirate the growth medium, wash cells with 1 mL of warm phosphate-buffered saline (PBS), and then fix the cells with 1 mL of 3.7% formaldehyde for 10 min.
3. Aspirate formaldehyde and stain cells in each well with 1 mL of 0.05% crystal violet for 30 min. Wash excess dye away using three washes of 1 mL deionized water.
   NOTE: Depending on the level of dye uptake, several additional washes may be needed to remove background staining to promote cell visualization.
    1. Using fine tipped forceps, retrieve coverslips and place them face down on a labeled glass slide with a drop of clear mounting reagent.

4. Under a light microscope, randomly choose 20 views of non-overlapping cells from each coverslip for the cell extension assay.
    1. Take photomicrographs at 400x magnification of each field of view. Directly count the number of each type of extension for each of the 20 representative cells.
       NOTE: As such, 20 isolated cells should be examined for each tested condition to ensure reliable results. Filopodia were defined by cellular extensions with a base of 5 microns or less and a single apex. Lobopodia were defined by cellular extensions with a base of 5 to 20 microns of length containing extensions with multiple apices. Lamellipodia were defined by cell extensions that are greater than 20 microns in length and with or without apices. Depending on the tested cell type, the sizes of cell extensions may differ and may require refinement of identifying parameters.

5. Determine the average number of each cell extension in each cell type (*i.e.*, SW-13, SW-Neo, SW-DDK) across the 6 wells examined.
6. Using a one-way analysis of variance (ANOVA) statistical test, compare differences in the average percentage of cell-membrane extensions among the different test cell types.

2. Adhesion Assay

1. Maintain SW-13, SW-Neo, and SW-DKK3 cells in a standard humidified incubator at 37.0 °C and 5% CO_2 in growth medium.
    1. Using a hemocytometer, count and plate 100,000 cells of SW-13, SW-Neo, and SW-DKK3 per well into separate 6-well plates and grow overnight in growth medium.
    2. Seed 6-well plates for each cell type tested, using one plate for each of the 6 designated time points tested below.
       NOTE: The time points may vary for different cell types.

2. After overnight incubation, wash cells with warm PBS once then add 0.5 mL of 1x non-enzymatic cell dissociation solution to each well.
    1. Swirl the plate to spread the cell dissociation solution. Aspirate the designated plate at defined time points (*i.e.,* 1, 2, 3, 5, 10, and 15 min) and then wash with 1 mL warm PBS solution three times.
    2. Between washes, gently tap the plates to detach loosely attached cells.

3. Aspirate any remaining PBS and fix the cells remaining attached to the plate with 1 mL of 3.7% formaldehyde for 10 min.
4. Stain cells with 1 mL of 0.05% crystal violet for 30 min then wash excess dye away with 1 mL of deionized water. Titrate wash to promote cell visualization.
   NOTE: Depending on the level of dye uptake, several additional washes may be needed to promote cell visualization.
5. Using a light microscope at 100x magnification, count the remaining attached cells in each well for each plate.
   NOTE: Use of transparent rulers or marking fine pen guides on the bottom side of the plate will help to avoid repeated counting of the same cells.
6. Determine the average number of attached cells per well for each plate.
7. Compare the rate of cell detachment between SW-13, SW-Neo, and SW-DDK3 cells over the given time period using a two-way ANOVA statistical test.

Representative Results

Using the above assays, the effects of DKK3 overexpression on cell extension morphology and cell adhesion properties were tested in the established ACC cell line SW-13, *in vitro*. Cells overexpressing DKK3 were generated by stably transfecting SW-13 cells with Myc-DDK tagged pCMV6-Entry/DKK3 plasmid vectors and designated as SW-DKK3. Similarly, cells stably transfected with empty vector pCMV6-Entry were created as a transfection and passaging control and defined as SW-Neo. SW-13 cells were used as an additional parent-cell control. Overexpression of DKK3 in SW-DKK3 was confirmed by quantitative real-time PCR and Western blot techniques[8].

Briefly, tested cells SW-13, SW-Neo, and SW-DKK3 were grown overnight in 6 well plates on cover slips. Cells were then fixed with formaldehyde and stained with crystal violet. Cell extension characteristics were then quantified under microscopy per the parameters described above. On analysis, SW-DKK3 cells displayed a more differentiated phenotype noted by increased lobopodia and multidirectional cell polarity (p=0.01, one-way ANOVA, **Figure 1** and **Figure 2**), suggestive of a lack of directional motility. In contrast, parental SW-13 and SW-Neo cells maintained polarity and displayed greater filopodia arranged in a planar orientation (p=0.01, one-way ANOVA, **Figure 1** and **Figure 2**)[8].

To determine whether increased lobopodia formation altered cell attachment properties, we performed a cell adhesion assay. Briefly, cells were grown overnight and cell detachment was performed with non-enzymatic cell dissociation solution at progressive time intervals up to 15 min. Cells were then fixed with formaldehyde, stained with crystal violet, and counted using a light microscope. SW-DKK3 cells showed a significantly greater attachment affinity compared to SW-13 and SW-Neo cells (p<0.01, 2-way ANOVA, **Figure 3**). These results indicate that DKK3 promotes lobopodia formation, which in turn promotes cell attachment[8].

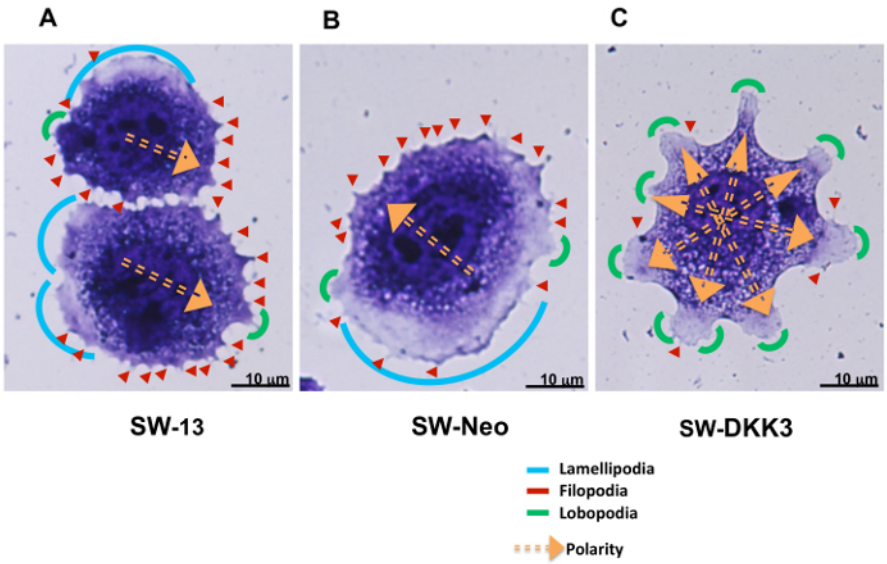
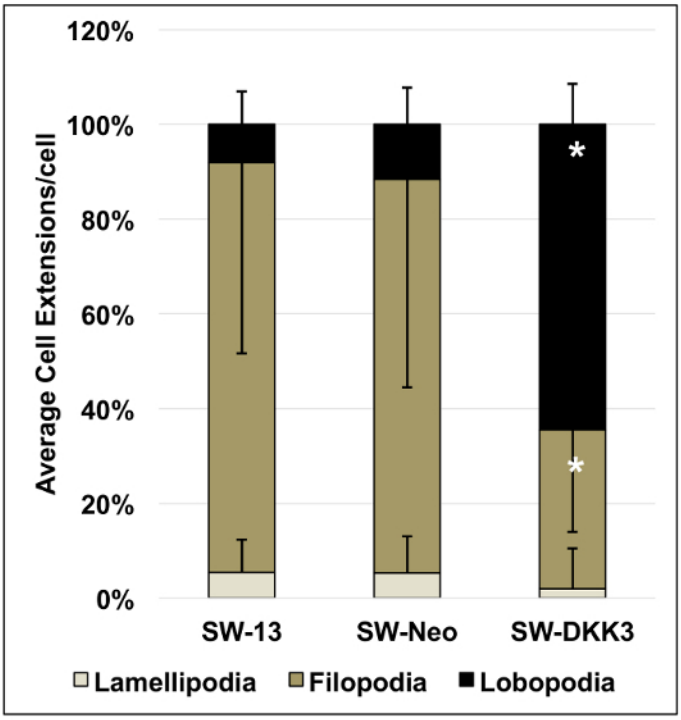
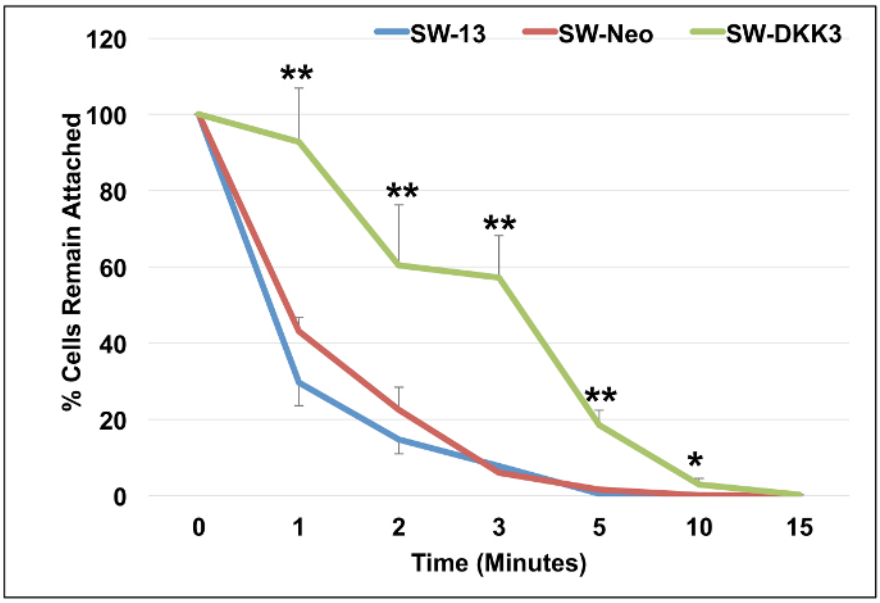


Figure 1: Enforced expression of DKK3 alters cell-membrane extensions. SW-13 **(A)**, SW-Neo **(B)**, and SW-DKK3 **(C)** cells were grown on glass coverslips, fixed with formaldehyde, stained with crystal violet, and imaged. Photomicrographs are taken using a light microscope at 400x magnification. SW-13 and SW-Neo cells formed mostly filopodia (red arrowheads) and lamellipodia (blue arc) cell extensions. In contrast, SW-DKK3 cells formed more lobopodia (small green arcs), with near absence of lamellipodia, and very few filopodia. While the SW-13 **(A)** and SW-Neo **(B)** cells appear to be polarized (orange arrows) with filopodia at the leading edge and lamellipodia at the lagging edge, SW-DKK3 cells **(C)** showed evenly distributed multidirectional lobopodia. Experiments were performed in duplicate. Please click here to view a larger version of this figure.



*p=0.01

Figure 2: DKK3 promotes lobopodia formation. The relative representation of filopodia, lobopodia, and lamellipodia per cell for each cell type were calculated by tabulating cell extensions on individual cells. Cells in twenty photomicrographs of randomly taken microscopic field of views (400x magnification) of SW-13, SW-Neo, and SW-DKK were used for quantification. Error bars indicated standard deviation and (*) indicates p<0.01 using a one-way ANOVA representing a significant increase in number of lobopodia in SW-DKK cells. A total of 20 cells were examined for each tested cell type and experiments were performed in duplicate. Please click here to view a larger version of this figure.



*p=0.01; **p=0.001

Figure 3: DKK promotes cell adhesion. One hundred thousand SW-13, SW-Neo, and SW-DKK3 cells per well of 6-well plates were allowed to grow overnight and were treated for detachment at designated time points (x-axis). The cells remaining attached were fixed, stained, and counted and the percentages of cells remaining attached were plotted (y-axis). Error bars indicate standard deviation and (*) indicates p<0.01 and (**) indicates p<0.001 using a 2-way ANOVA representing a significant increase in SW-DKK cell attachment strength. Each experiment started with 100,000 cells and experiments were performed in duplicate. Please click here to view a larger version of this figure.

Discussion

Here we describe an *in vitro* quantitative method to characterize cell extensions with ease, few pit-falls, and reliable reproducibility that can be applied for various test conditions. Moreover, simple, quantifiable adhesion and/or motility assays can be performed simultaneously to correlate potential functional significance of observed alterations in cell membrane extensions.

However, these methods may present some potential limitations. First, the criteria specified here to identify different types of cell extensions are based on adrenocortical SW-13 cell morphology and hence likely need refinement in classifying parameters when applied to other cells types and/or experimental conditions. Further, in extended time-course experiments, the potential influences of cell membrane extension alterations on the cell cycle and cell death need to be considered.

Second, the protocol may rely on potential individualized calling by the examiner, which could limit the assay's reproducibility due to inter-observer variation. The high number of cells that are examined for each test group likely overcomes part of this bias; specifically, when 20 cells are examined for each assay. Inclusion of multiple individuals to perform extension calling in a blinded fashion is suggested to mitigate the potential subjective calling bias.

Finally, care should be taken when associating changes in cell extension class switch with alterations in attachment properties, where a simplified version of adhesion properties could be the outcome of altered preference for attachment substratum rather than the altered affinity for the tested substratum. Coupling the cell extension class switch with migration or invasion in the context of various extra-cellular matrix components could clarify the observed adhesion properties.

In conclusion, the protocol outlined here provides a simple and reliable method for measuring the functional significance of cell membrane extension switch under various test conditions.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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