**Reduced expression of PNUTS leads to dephosphorylation of the tumor suppressor protein Retinoblastoma (Rb) through phosphatase activation and caspase-mediated apoptosis in MCF7 breast cancer cells in a three-dimensional culture**

**Running Title: Rb dephosphorylation by PNUTS Knockdown in 3D**

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**Abstract:**

The tumor suppressor Retinoblastoma (Rb) protein is considered important in controlling cell proliferation and apoptotic cell death in all cells in conjunction with specific cyclin-cdks complexes, p53, another tumor suppressor protein, and caspase 8 inhibitors, enzymes that change the morphology required for apoptosis.1 In PNUTS (Phosphatase Nuclear Targeting Subunit) knockdown experiments, a cascade of events occur starting with dephosphorylation of Rb through Protein Phosphatase 1 (PP1), dissociation of a family of transcription factors (E2F1) from Rb, and ending with caspase-8 mediated apoptosis. These results have been shown in 2-D environments but have not yet been explored in 3-D cultures which ties into the importance of this study. In this study, 3-D models were used to explore cultured MCF7 breast cells to see how the cells receive and respond to cell division signals from their surrounding environment in comparison to the results in 2-D cultures. The use of 3-D models will lead to advances in more defined results relatable to understanding the activity of real life cancerous cells. In addition, PNUTS Knockdown does reduce cell number through apoptosis in the 3-D cultures just as revealed in the 2-D cultures. Furthermore, these results reflect the overall importance of this study in relation to cancer research which is the incorporation of dephosphorylated Rb by PNUTS Knockdown in order to cause cancerous MCF7 breast cells to undergo apoptosis in 3-D environments.

**Introduction:**

 Cancer is a prevalent disease in the world today, therefore understanding the causes and molecular makeup is significant to clinical applications. Approximately 1 out of every 8 women will be diagnosed with breast cancer in her lifetime.3 Uncontrollable cell division defines cancer. The behavior and growth of carcinomas varies and can rapidly metastasize or aggressively develop over the course of a few years.3 Many components have a role in cellular development in normal cells such as growth factors, hormones, molecules, and proteins.3 The tumor suppressor protein Retinoblastoma (Rb) is an essential regulator of cellular processes involved in carcinogenesis including cell proliferation and apoptosis.¹ Many factors contribute to the activity of Rb such as cyclin dependent kinases, protein phosphatase 1 (PP1), members of the E2F1 family (a family of transcription factor proteins), and Phosphatase Nuclear Targeting Subunit (PNUTS).1 Cdks especially are important in the Rb pathway leading to excessive phosphorylation of Rb, a trait that is observed in all cancer types. Understanding the importance of Rb and its phosphorylation pathways is essential to identifying experimental techniques to manipulate these pathways and potentially develop clinical therapies.

 In order to understand changes in Rb in regards to division of cancer cells, the process of how normal cells divide is important to be familiar with. For normal cells, cell division is tightly regulated so that cells do not continuously divide.4 External and internal signals must also be met before division begins to help regulate the cell cycle which are a sequence of events that repeatedly prepares a cell to replicate and divide to form two daughter cells.4 A cell goes through the stages of the cycle which are Interphase (consisting of G1, S, and G2) and Mitosis/Cytokinesis.4 During Interphase, a cell receives a growth factor signal to prepare to grow and synthesizes proteins, lipids, and carbohydrates needed for the cell to continue through the cycle (G1 phase), then DNA is replicated (S phase) followed by additional growth and metabolism (G2 phase).4 Checkpoints in between each phase help regulate the movement of cells through each phase by internal and external signals.4 Then mitosis occurs which evenly divides chromosomes and splits the cell into two identical daughter cells.

Important components that regulate the cell cycle are cyclin-cdk complexes. Cyclin levels are low at the beginning of the cell cycle but will gather in higher concentrations to bind to cyclin-dependent-kinases (cdks) to form complexes.4 These complexes become enzymatically active to stimulate additional proteins and allow cell progression from one phase in the cycle to the next.4 Specific cyclin-cdk complexes regulate each checkpoint in the cycle to either allow stimulation of the cycle for cell division to occur through a positive growth signal or stalling of the cycle to inhibit the division of the cell through a negative growth signal.4 The more important Cdks that play a role in the cell cycle are Cdk4 and Cdk6 (G1 phase) and Cdk2 (beginning of S phase).5 Normally, in response to a growth factor stimulation, Cdk4/6 bind to cyclin D to initiate the phosphorylation of Rb.5 Usually, quaternary complexes are formed by cyclin kinases by acquisition and attachment of two additional subunits.7 An additional subunit also involved in this pathway is the tumor suppressor protein p53 which is actually mutated in over half of all cancers.4 Under normal conditions, when there is damage to DNA, p53 is activated in order to repair the damage or cause the cell to undergo cell cycle arrest.6 p53 is considered the cellular gatekeeper for growth and division which in turn prevents formation of cancers.6 Additionally, it works in conjunction with p21, which is a cyclin-dependent kinase inhibitor (CKI) protein, that inhibits cyclin kinases which in turn halts cell proliferation due to overexpression in cells around the G1 and S phases.7 Affiliation with all of these cyclin-cdk complexes secures the importance of Rb in the cell cycle.

In addition to cell proliferation, Rb is important in apoptosis. Apoptosis is programmed cell death or cellular suicide.4 Cancer cells often avoid apoptosis due to the fact that they can divide without growth factors present.4 Mutated positive signal proteins which are encoded by oncogenes allows unregulated control of the cell cycle to occur thus leading to a rapid production of cancerous cells.4 Moreover, cancer cells have the ability to invade neighboring healthy cells and sharply increase their growth factor expression to support the rapid metastasis of the cancer cells.4 In order for these cancerous cells to undergo apoptosis, a heavy technique must be placed upon these cells to “go against their nature”, which essentially involves Rb. Rb dephosphorylation has been widely observed during apoptosis. Normally, Rb is phosphorylated in cells and follows its specific metabolic pathway involving Cdk4/6 and cyclin D resulting in cell proliferation. In order to alter an inactive Rb to an active Rb, dephosphorylation, or the removal of a phosphate, must occur in order to allow active Rb to act as a brake for cell division. Once dephosphorylated, Rb dissociates from its bound E2F1 transcription factor family and essentially apoptosis is induced within the cells. One important member missing from this equation is PP1, which helps regulate Rb phosphorylation. It is known that when PNUTS, a PP1 targeting subunit, is bound to PP1, it inhibits PP1 activity with Rb phosphorylation sites.1 However, when cells undergo stress, this allows PNUTS to dissociate from PP1 which then can allow dephosphorylation of Rb at specific binding sites followed by E2F1 dissociation and caspase inhibited interactions.1 The activation of phosphatase PP1 is essential in understanding its relevance to apoptosis through dephosphorylated Rb.

In order to detect morphological changes in the MCF7 cells as well as the function of Rb phosphorylation, a 3-D model was used. Even though the use of 3-D epithelial cell cultures is a fairly new technique, its contribution to research is mostly beneficial. Firstly, an in vitro 3-D culture model consists of a more complex structure that resembles a real-life in vivo tumor environment as well as allows for a better understanding of the molecular and cellular mechanisms.8 In addition, 3-D models allow researchers to perform genetic manipulations and observe the biological changes that occur from the genetic alterations.9 Disruptions of the architecture of breast cells can be observed through a 3-D model as well.9 A breast cell structure is molded through morphogenesis and forms clumped, glandular like areas at the ends of the breast ducts containing a hollow lumen called mammary acini.9 Due to this intricate structure, densities and compositions of the tumor can be controlled in a 3-D model and serves as a differentiated and versatile reference to the physical differences in breast tumors and tissues alike.8 These models allow control over composition and reduced time efforts for the cancer studies as a whole. 3-D culture systems are currently being utilized for all different types of cancer research due to the fact that these models can be useful for screening the activity of potential cancer therapies and drug efficiency.8,9 In regards to this study, results from a 3-D cell culture will support the importance of Rb in in-vivo breast cells.

Previous studies have provided insight on MCF7 breast cancer cells grown in 2-D cultures that lead to activated Rb phosphatase in the cells. Through PNUTS siRNA, PNUTS was able to be diminished to permit increased phosphatase activity towards Rb thus resulting in dephosphorylated Rb. As a result, E2F1 transcription factors were released from Rb and caused caspase mediated apoptosis in the cells. Moving forward with the positive results from the 2-D cultures, progression can be made with the use of 3-D MCF7 cell cultures to investigate the function of Rb phosphatase and its control of cell proliferation and apoptosis.

**Materials and Methods:**

Part I: 3D-Cell Culture of MCF7 cells

After mastering the techniques used in 2-D cultures, progression to the next step was possible which is working in 3-D environments with MCF7 breast cells. Before the administration of PNUTS siRNA can occur, the proper techniques of working with 3-D cultures must first be practiced. Understanding the importance and use of 3-D cultures and their needed materials is essential in this experimentation. First, knowledge of the actual culture and media used is important for 3-D’s. A special extracellular matrix material of collagen and laminin, called Matrigel,10 in conjunction with a growth media is specifically used in our 3-D cultures in order to cause the MCF7 cells to form 3D structures. Matrigel was kept frozen until use, then thawed, and kept on ice. The Matrigel-cell-media mixture that is constructed during the experiment is dispensed into 8-chamber slides.10 Aside from the assay media and Matrigel, aliquots of EGF (Epidermal Growth Factor) are also introduced to guide the growth of the cells in the cultures. The cells in the slides must also be fed with a media-diluted EGF-Matrigel mixture every four days.10 The purpose of the Matrigel is to mimic extracellular matrix in which the cells form 3D structures. During the incubation periods, MCF7 cells should begin to form clumps which resemble tumor-like structures, which are the true structures of breast cells in the human breast.

Part II: Protein Extraction

 The purpose of protein extraction in this experiment is to release the protein from the cells. To perform this procedure, the first main focus is to dissolve the Matrigel in our samples. The basic steps are aspiring media from each chamber in the slide, washing the chambers with PBS, adding 300 microliters of cell recovery solution to make a slurry out of the substance plus Matrigel in the slides and transferring to eppendorf tubes, and incubating the tubes on ice for one and a half hours to fully dissolve the Matrigel. Then, the tubes are placed on the refrigerated centrifuge at 4°C at 6000 rpm for three minutes. When the tubes are removed, check the pellet, however if it is still a jelly-like substance, the contents must be re-suspended and incubated for a longer period of time. Once no jelly is present, the pellet must be re-suspended in PBS, spun down for a minute at 6000 rpm at 4°C, and re-suspended again in made-up cell lysis buffer for 25 minutes on a rocker at 4°C. Next, the pellets must be spun in the centrifuge at 4°C for 10 minutes at 13000 rpm. The supernatant is kept for the next portion and the pellet can be thrown away.

Part III: Cell Lysis and Protein Concentration Assays

 If you have a frozen sample from before, allow the pellet to thaw and remove excess liquid with a Pasteur pipette. 400 microliters of supplemented lysis buffer (containing protease inhibitors Aprotinin and PMSF and a 10x Cell Signaling Tech Lysis Buffer) is added and the cells were transferred to an eppendorf tube while being kept on ice at all times. The tube is then rocked at 4°C for 10-15 minutes and then centrifuged at high speeds at 4°C for 10 minutes. Remove the liquid supernatant, which is now your protein sample, and while on ice, another tube is placed to use for the protein concentration assay. Six glass test tubes are set up labeled 0,2,4,6,8, & 10 plus one for each protein lysate for testing and add 800 microliters of DI water to each tube. Carefully add 0,2,4,6,8,10 microliters of 1mg/ml BSA solution to standards, about 1-5 microliters for each sample, and 200 microliters of Biorad dye to all tubes. Tubes are then mixed by vortexing and then placed in the appropriate cuvettes to be used in the BioRad Spectrophotometer which will perform the standard curve and protein concentration assay to see how much protein is in the sample.

Part IV: Gel Electrophoresis/ Western Blotting

The purpose of gel electrophoresis in this experiment is to separate the proteins by size with the largest sequences at the top and the smallest towards the bottom of the gel. Precasted gels with specific percentages are used as well as the BioRad gel power pack to ensure the running of the gel. In order to visibly see the resulting bands from the gel and later reconstruct them to detect the Rb protein, the technique of a western blot is performed. Western Blot or immunoblotting is a routine technique for protein analysis used to positively identify a specific protein in a complex mixture in order to obtain qualitative and semi-quantitative data about the protein.² There are multiple steps involved in the blotting which consists of separating macromolecules using gel electrophoresis (as described above), transferring of materials onto a specific membrane, blocking of the membrane to prevent nonspecific binding of antibodies to the membrane surface, washing, and finally an introduction of an enzyme-labeled probe or dye in order to undergo detection by chemiluminescence. In this experiment, a special membrane is used called nitrocellulose paper which is set up in the following order: filter paper, gel, nitrocellulose paper, and filter paper. The purpose of the multiple layers is to be able to safely transfer the band sequences from the gel onto the nitrocellulose paper with the assistance of heat. The nitrocellulose paper is then treated with block solutions in order to reduce non-specific antibody interactions on the band sequences. During this experiment, primary and secondary antibodies are utilized, the primary being the mouse monoclonal antibody and the second an anti-mouse which both will help lower the background interference.² Peroxidase has an important use in that its used as a label for protein detection and will catalyze the oxidation of substrates which results in light production.² Once washing occurs which wipes away unbound reagents, chemiluminescence is used to detect the Rb protein on the blot. Through this chemiluminescent detection, we will be able to detect if any phosphorylation activity occurred with Rb in the sample.

**Results:**

**Effect of PNUTS Knockdown on apoptosis**

Much research prior to this experiment provided apoptotic results in 2-D cultures, however the majority of my experiment was carried out in 3-D cultures and some in 2-D to be utilized as a comparison for my new results. In the 2-D cultures, there is an evident difference in the number of cells between the PNUTS KD (Seq5) and the Staurosporine wells (Figure 1). The exponential numbers provided by the GloMax plate reader are shown in Figure 1 in combined and average numbers. Again, PNUTS KD (RNAi) is the experimental group in the experiment while Staurosporine is known to kill off cells thus considered a positive control for this experiment. The Staurosporine that was added to the Hs578T cacner cells and left for 48 hours induced the most apoptotic activity considering that it is one of the positive control lanes of the plate. PNUTS KD (Seq 5) also resulted in induced apoptosis but significantly less than the induction by Staurosporine, the positive control. Multiple t-tests were performed to gather p-values in order to statistically support the results. All p-values are shown in Table 1 below. An unpaired, two-tailed t-test was used because there are a lot of factors that play into the growth overtime of the cells even though all the same Hs578Ts cells were used. As a result of Table 1, all of the p-values are below 0.05 which proves them to be statistically significant. Even the t-test between the NT and the High Seq 5 provides additional support in that the results point towards a real contribution of PNUTS in apoptosis.

Figure 1: Results of Cell Titer Glo- Assay with Chemiluminescent Reagent 2 in GloMax plate reader by Promega. The cells in this assay were Hs578Ts originally grown in 2D cultures and plated over a week time span. 48 hour Staurosporine seemed to have the most effect on induced apoptosis of the cells compared to PNUTS KD (either high or regular Seq 5).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| CT vs NT | CT vs Seq 5 | CT vsHighSeq 5 | CT vs 48St | CT vs 24St | NT vsHigh Seq5 |
| 7.03593e-09 | 6.493e-03 | 8.55882e-16 | 2.33957e-16 | 9.77635e-17 | 1.38456e-05 |

Table 1: Unpaired, 2-tailed t-tests performed on all lanes in plate compared to the control (CT) lane and one of a comparison of NT vs High Seq 5 to help further statistically prove the results.

**Effect of PNUTS KD in 3-D Cultures**

Apoptosis in MCF7 cells was determined through immunofluorescence microscopy. The cells cultured in the 3-D Matrigel environment began clumping into expected acinar-like structures in the slides. A fluorescent dye was added to track the specify of the cells and Rb after PNUTS KD was induced in the culture. The dye marks the cells blue and phosphorylated Rb in green. As shown in Figure 2, approximately 70-80% of the MCF7 cells undergo apoptosis due to the presence of less green fluorescence in the culture. This estimated reduction in cell number suggests that PNUTS KD does allow Rb dephosphorylation leading to mediated apoptosis in both 2-D (data not shown) and 3-D cultures.



Figure 2: MCF7 cells were cultured in a Matrigel (3-D) environment and given time to clump thus resembling real life acinar-like structures. MCF7 cells were dyed with a green immunofluorescence [Phospho-Rb antibodies] in order to monitor the amount of phosphorylated Rb in a Non-targeting sample compared to a sample that had PNUTS KD initiated in order to induce apoptosis. The presence of less fluorescence proves that Rb was indeed dephosphorylated in order to knock out PNUTS from the protein thus permitting apoptosis in the 3-D culture.

**Evaluation of the Rb protein and the Western Blot**

Now that we have shown that PNUTS KD can induce apoptosis in the 3-D MCF7 cells, we investigated the role of Rb activity. The dephosphorylation of Rb was evaluated via a Western Blot in order to properly distinguish whether or not the protein is modified in any way during the experiment. The general antibody used to detect Rb on a blot is a monoclonal Anti-Rb antibody. Immunoblotting results are compared with the standard Precision Plus Protein ladder used during gel electrophoresis. The ladder exhibits a column of bands all consisting of different weights measured in kilodaltons (kD) used as a comparable reference for the weight of Rb bands on a blot, which should surface around 110 kD. Below is a photo for the standard ladder and how it looks on different mediums.



Precision Plus Protein Dual Color Standard from BioRad. Runs from 10-250 kD with 10 recombinant proteins and two differently colored stains for reference.

After the western blot is performed, the appearance of bands is evaluated. In Figure 3, which was carried out with 2-D MCF7 cancer cells, the loss of bands in the second columns for the Rb row proves dephosphorylation of Rb. Lesser upper bands for Rb indicated apoptotic activity occurred within those cell lines. The appearance of bands will be lesser on the second columns for Rb due to the fact that at the conclusion of the experiment, Rb is dephosphorylated which in turn reduces the amount of protein detected by the blot. The darker the bands, the more protein that was picked up by the blot at that weight. Cleaved Parp and β-actin are used as markers for apoptosis in samples to support whether or not apoptosis occurred.11



Figure 3: Results of Western Blot as performed in Materials and Methods. MCF7 cells undergone UV stress and treated with Roscovitine or DMSO for 24 hours before immunoblotting. Dephosphorylation of Rb is displayed by the migrating, smaller or loss of bands in the second lane of the Rb row. Cleaved Parp and β-actin expression was essential to confirm induction of apoptosis and equal loading of protein in the cultures.

Figure 3 shows that about 60% of the Rb protein was lost due to UV stress and about 45% was lost due to treatment with Roscovitine, which is a selective inhibitor of cyclin-dependent kinases considerably used as a positive control to induce apoptosis resulting in an accumulation of dephosphorylated Rb. Due to the presence of fewer, lighter bands for Rb, it is safe to conclude that under times of cellular stress and other various apoptotic stimuli, Rb becomes dephosphorylated.

Although 3-D cultures are exceedingly more complex than 2-D cultures, the mechanism behind Rb activity is the same. Even though the structure of cells may have been an altered factor of the experiment, the same expected results of dephosphorylated Rb were demonstrated through immunoblotting providing us with significant quantitative results on apoptosis in the MCF7 cancer cells.

**Discussion:**

The results of this study indicate that knockdown of PNUTS by PNUTS siRNA results in Rb dephosphorylation and subsequently causes apoptotic cell death. As shown in previous research, the binding protein PNUTS is associated with a specific phosphatase called PP1 (Protein Phosphatase 1). Under stressful conditions, PNUTS dissociates from this complex which allows PP1 to interact with inactive Rb thus dephosphorylating it to become active Rb.¹ The dephosphorylation of Rb allows interaction with E2F transcription factors to cause caspase mediated apoptosis in cells.¹ E2F1 is the specific member of the E2F family of transcription factors that regulates the expression of genes involved in several actions such as apoptosis, differentiation, cell proliferation, and development.¹ Caspase-8 is within a caspase family of enzymes which are responsible for apoptosis execution.¹

Using what was known on the use of PNUTS RNAi and Rb active and inactive states, we experimentally carried out projects in both 2-D and 3-D cultures. We used the results from the 2-D cultures which concluded that the PNUTS RNAi does indeed kill breast cells, and incorporated it into 3-D cultures. However, to monitor the presence of Rb, Western Blots were used for the 3-D cultures whereas in the 2-D cultures, the 96 well plates and GloMax plate reader expressed the results of cell death. Understanding the differences between the 2-D and 3-D cultures is important in that cancer develops in a 3-D environment in real life, not in 2-D. Therefore utilizing a more realistic model to understand cell proliferation and apoptosis in breast cells is essential in better understanding cancer and metastasis in vivo.

The use of 3-D cultures is still a new technique being explored for research, however it is already exemplifying advantages, as previously mentioned earlier. Some recent cancer research is utilizing 3-D model systems that mimic real life models.8,9 Another popular model being utilized is the in-vitro 3-D model system which portrays a more appropriate organization of breast tumor tissues and displays cellular results in a form that portrays the environments that cells really live-in vivo.8 There is more control with the use of these in-vitro models as well as a more accurate portrayals of variable sizes, densities, and shapes of human breast tumors (compared to the 2-D models).8 The use of these 3-D models decreases the limitations that researchers seemed to come across while using 2-D cell cultures such as incomparable structure to the real life tumor. Additional research using 3-D models, which are more realistic than 2-D models, could lead to major breakthroughs for cancer researchers in the future

Furthermore, even though the overall importance of Rb in apoptosis and cell cycles is still being investigated, this project collaborates years of past findings from other researchers. In several cancers, Rb is often found to be highly phosphorylated which deems it as a reasonable targeting molecule to dephosyphorylate.1 Additionally, other studies show that PNUTS knockdown induced apoptosis and was found to be Rb dependent. This statement means that PP1activation is only towards Rb and no other substrate, deeming Rb an important factor in apoptotic conditions.1 Therefore, with a continued focus on the Rb protein as well as its activity in 3-D cultures, important discoveries can be made on how to reverse phosphorylation of Rb in order to cause apoptosis in various cancers, including breast cancer.

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