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14. ABSTRACT The altered balance in the expression of PKC isozymes is a distinguished feature of cancer. One of the most notable alterations in epithelial cancers is the upregulation of PKCε. This kinase has emerged as a potential oncogene and tumor biomarker, however, little is known regarding a potential causality between its upregulation and cancer development. In this research we wished to understand the role played by PKCε in prostate cancer cells and establishes the proof of principle of PKCε inhibition as a putative therapeutic strategy. Using a RNAi approach we found that PKCε inhibition decreases size of tumor generated by PC3-ML cells in athymic/balb-c mice. Accordingly, immunohistochemical analysis of xenografts 10 days after inoculation showed a marked induction of cell death in PKCε depleted cells. In addition we found that this kinase may be relevant in attachment, anchorage-dependent and anchorage-independent growth of PC3-ML cells. Preliminary data showed that PKCε depletion markedly impaired the ability of PC3 to migrate and PC3-ML to invade and migrate. In summary, our results argue for a role of PKCε in prostate cancer development and metastasis, highlighting its potential as a therapeutic target.					
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INTRODUCTION

The main goal of our research supported by DOD is to elucidate the mechanisms by which protein kinase C (PKC) epsilon is implicated in tumorigenesis or metastatic events in prostate cancer. The PKC family comprises 10 different isozymes, which have been classified into 3 groups: “classical” or calcium dependent PKCs (cPKCs α , β I, β II, and γ), “novel” or calcium-independent (nPKCs δ , ϵ , η , and θ), and “atypical” (aPKCs ζ and λ/ι). Members of the first two groups can be activated by phorbol esters, natural compounds originally described as tumor promoters. cPKCs and nPKCs are the most prominent targets for the lipid second messenger diacylglycerol (DAG), a lipid generated mainly by phospholipase C isozymes upon activation of tyrosine kinase and G-protein-coupled receptors (GPCRs). Activation of these receptors leads to DAG generation, which triggers the translocation and activation of cPKCs and nPKCs. PKC isozymes modulate important signaling pathways that control proliferation, differentiation, survival, apoptosis, and malignant transformation. While PKC has been initially viewed as a growth-promoting kinase, it became clear particularly in the last decade that PKCs could trigger growth inhibitory or apoptotic responses as well (1, 2, 3). Despite extensive investigations, a challenge has been to establish the relative contribution of individual PKC isozymes to cancer progression, a complex task due to the vastly different roles that PKC isozymes play and the fact that these vary according to cell type. Most prostate cancer cell lines express cPKC α , nPKC δ and nPKC ϵ . The roles of individual isozymes in mitogenesis and survival of prostate cancer cells are still a subject of debate.

Using pharmacological and molecular approaches, our laboratory established an essential role for PKC δ and PKC α in prostate cancer cell apoptosis. However, one of the most relevant and yet poorly understood member of the PKC family is PKC ϵ . This kinase has been shown to act as a dominant oncogene in different cellular models (4, 5, 6, 7) and it has been implicated in neoplastic transformation and progression to a metastatic stage in NIH 3T3 fibroblast (4, 8). PKC ϵ is highly overexpressed in human prostate cancer. Nearly 100% of human prostate tumors overexpress PKC ϵ , particularly advanced metastatic tumors. Evidence linked PKC ϵ to Akt and survival in various cancer cell types (9, 10). In prostate cancer cells PKC ϵ functionally interacts with Bax and Akt to promote survival (11), activates the Stat3 pathway (12), and is implicated in the transition to androgen independence. Verma’s group has recently demonstrated that genetic deletion of PKC ϵ in TRAMP mice down-regulates prostatic Stat3 activation and Stat3-regulated gene expressions and inhibits prostate cancer development and metastasis (13). A study from our lab found that PKC ϵ promotes survival in prostate cancer cells by modulating Bad phosphorylation and the secretion of pro-apoptotic death factors (14). In addition, using a transgenic mouse model our lab established that overexpression of PKC ϵ in the prostate confers pre-neoplastic lesions and cooperates with Pten deficiency.

A main goal of our research is to understand the role played by PKC ϵ the proof of principle of PKC ϵ inhibition as a putative therapeutic strategy.

BODY

1. Generation of PKC ϵ -depleted cells in prostate cancer cell lines: We established cultures of PKC ϵ -depleted cells in the following prostate cancer cellular models: PC3, PC3-ML and DU-145. Silencing was achieved by means of infections with shRNA lentiviruses from Sigma). Selection was made for at least 3 weeks in presence of puromycin (0.3 μ g/ml). Cultures were made with 5 different shRNA target sequences (#844, #845, #846, #847, #848). Levels of PKC ϵ showed that the highest PKC ϵ depletion was achieved in cells infected with sequences number #844 and #848 in all prostate cancer cells used (Fig. 1).

2. PKC ϵ -deficient prostate cancer cells have impaired tumorigenic capacity: First, we carried out a preliminary assay injecting different amounts of PC3, DU-145 and PC3-ML cells to determine the cell concentration and kinetics of the tumor growth in our experimental conditions. We injected 1×10^6 , 3×10^6 and 5×10^6 or 8×10^6 cells suspended in 100 μ l of PBS in the flank of at least 3 athymic BALB/c nude mice. Tumor growth was determined every 3 days beginning at the first signs of tumor appearance. Tumors formed by PC3 and PC3-ML cell lines showed a high correlation between number of cells and size of the tumor. We could not establish a solid correlation with DU-145 cells, and therefore we focused on the other two cell lines (Fig. 2a).

In the next experiment we injected 4×10^6 PKC ϵ -depleted PC3 cells (sequences #844 and #848) per animal (n=10) or cells infected with non-target control (NTC) shRNA lentivirus. We used a caliper to measure tumor cross-section area formed by PC3 cells every 3 days, and we did not detect any significant difference in size between tumors formed by PKC ϵ -depleted cells or NTC cells during the duration of the study (Fig 2b). As a second approach we injected 4×10^6 PC3-ML cells of PKC ϵ -depleted cells (sequences #844 and #848) per animal (n=10) or the corresponding non-target control (NTC). We observed a significant difference between the PKC ϵ depleted cell and control cells (NTC) in this experiment (Fig 2b). As a third approach we decided to inject less number of cells. This time we injected 1.2×10^6 PC3-ML PKC ϵ depleted cells (sequences #844 and #848) per animal (n=10) or the corresponding non-target control (NTC). As we expected, we observed again a significant difference between the PKC ϵ -depleted cells and control cells (NTC) in this experiment (Fig 2b).

3. Characterization of proliferative and apoptotic properties in vivo and in vitro:

Histological analysis was carried out in tumors formed by PC3 cells at the end of the study (34 days). We could not detect significant differences in apoptosis by TUNEL. We also carried out an histological analysis of tumors formed by PC3-ML cells at the end of the study (24 days), and surprisingly we did not detect significant differences between tumors formed by NTC or PKC ϵ -depleted PC3-ML cells. We decided to analyze the incidence of apoptosis by TUNEL early during tumor formation. Immunohistochemical analysis of xenografts 10 days after inoculation showed a marked induction of cell death in PKC ϵ -depleted cells, as evidenced by a large number of TUNEL-positive cells (Fig. 2c, right panel).

In a different set of studies we found a reduced ability of PKC ϵ -depleted cells to attach (Fig 3a). Moreover, as shown in figure 3b, proliferation was significantly reduced in those cell lines in which PKC ϵ was stably depleted.

Growing tumors usually are under highly metabolic rates. Tumor cells are frequently exposed to stress, and that could determine the balance between survival or death. Preliminary data from our lab shows that PKC ϵ -depleted cells have similar levels of activated Akt and Erk, and therefore we hypothesize that other events could be responsible of smaller size of tumor generated by PKC ϵ -depleted cell. Cell death by apoptosis observed during the initial steps of

tumor formation in PKC ϵ -depleted cells together with decreased proliferation of these cells may be responsible of difference in size of tumor observed in this experiment (Fig. 2c, left panel). To gain further insight on the role played by PKC ϵ in the stress response of prostate cancer cell lines we decided to determine the role of PKC ϵ in stress-related cell death. We were unable to detect any significant differences in stress-related cellular death in response to osmotic or ER stress, but surprisingly we detected significant differences in response to oxidative stress. Figure 4a shows less survival rates in PKC ϵ -depleted cells in response to oxidative stress. In accordance with this result we detected higher ROS accumulation using DCFH as a probe (Fig. 4b) in PKC ϵ -depleted cells after exposure to a highly oxidant environment. This result suggests that PKC ϵ depletion could sensitize prostate cancer cells to oxidative stress.

4. PKC ϵ -deficient prostate cancer cells have impaired anchorage-dependent and anchorage-independent growth: We decided to establish the role of PKC ϵ in anchorage-independent growth in prostate cancer cell lines by measuring colony formation in soft agar. Briefly, 1×10^4 PKC ϵ -depleted PC3-ML cells or their corresponding controls (NTC and parental) were suspended in 0.37% agar and plated in triplicate onto a 0.7% bottom agar layer in 6-well plates. After 2-3 weeks the plates were stained with MTS and the number of colonies per dish was counted. PKC ϵ -depleted cells generated a similar number of colonies, but the size of these colonies was significantly smaller than NTC or parental (Fig 5a). Next, we analyzed the role of PKC ϵ in anchorage-dependent prostate cancer cell growth. Cell anchorage to substratum reflects the interaction of the ECM with integrins and cadherins and is strongly linked with specific events such as the expression of cyclins and cell cycle progression. Several reports in the literature have established a role for PKC ϵ in cell cycle progression (15, 16). We speculate that silencing PKC ϵ could have an impact on anchorage-dependent growth. PKC ϵ -depleted PC3-ML cells or their corresponding control were synchronized in G0 and plated on cultured cell dishes. After 2-3 weeks plates were stained with Methylene Blue, number and size of colonies were determined. As shown in the figure 5b, PKC ϵ depletion caused a significant inhibition in the number and size of colonies, pointing out to a role of PKC ϵ in anchorage-dependent growth in prostate cancer cell lines.

5. Overexpression of PKC ϵ in non-transformed prostate cells: We overexpressed PKC ϵ in RWPE-1 cells, a model used as “normal” non-transformed epithelial prostate cells using (ViraPower lentiviruses containing a Human PKC ϵ construct cloned into pLenti6/V-5 DESTRWPE-1). Selection was made for at least 3 weeks in the presence of blasticidin (0.3 μ g/ml). High levels of PKC ϵ were detected in these cells (RWPE-PKC ϵ) by Western blot (Fig. 6a).

6. PKC ϵ overexpression increases proliferation of RWPE-1 cells: RWPE-PKC ϵ display high proliferative rates compared to control cells (Fig. 6b), as determined by Crystal Violet staining. We plan to determine and quantify proliferative properties by cell counting, [3 H] thymidine incorporation, as well as cell cycle progression by flow cytometry.

7. PKC ϵ depletion affects migration and invasiveness: Migration assays in PC3 and PC3-ML cells were carried out using Boyden chambers (fig 7a). Cells (2×10^4) were seeded in the upper compartment of the Boyden chamber, and FBS was used as chemoattractant. We used 12 μ m polycarbonate membrane to separate the upper and lower chambers. PKC ϵ depletion markedly decreased the migratory properties of PC3 cells. The effect on PC3-ML was not so striking. Due to the highly metastatic properties of this cell line, we decided to

evaluate invasion through Matrigel. Fig. 7b shows that PKC ϵ depletion strongly decreased invasiveness in PC3-ML cells.

In addition, we examined if PKC ϵ overexpression in RWPE-1 cells confers a migratory phenotype. RWPE-1 or RWPE-PKC ϵ cells (2×10^4) were seeded in the upper compartment of the Boyden chamber, and we used EGF and pituitary bovine extract as chemoattractant. 12 and 8 μ m polycarbonate membrane were used to separate the upper and lower chambers. Neither migration nor invasion (using Matrigel) could be detected in RWPE-1 or RWPE-PKC ϵ cells after 24 h.

8. PKC ϵ -depletion does not affect activity of proteolytic enzymes in basal conditions: A preliminary experiment was carried out to evaluate the effect of PKC ϵ depletion on the activity of MMP-2 and MMP-9. Conditioned medium (CM) from PKC ϵ depleted PC3-ML cells or their corresponding controls were collected and MMP-2 and MMP-9 activities were determined by zymography on gelatin-impregnated gels. No differences were detected in basal conditions (Fig. 8). We plan to check MMP-2 and MMP-9 activities after stimulation with different cytokines and osteoclast conditioned medium (PC3-ML cells metastasize to the bone).

KEY RESEARCH ACOMPLISHMENTS

We successfully knocked down PKC ϵ in DU-145, PC3 and PC3-ML cells.

We successfully overexpressed PKC ϵ in RWPE-1 cells.

We determined that PKC ϵ is involved in the invasion properties of PC3-ML and PC3 cell lines.

We found that PKC ϵ depletion decreases the size of tumor generated by PC3-ML cells in athymic/balb-c mice.

We found a marked induction of cell death in PKC ϵ -depleted cells upon inoculation into nude mice.

We found that PKC ϵ is implicated in attachment, anchorage-dependent and anchorage-independent cell growth of PC3-ML cells.

CONCLUSION

The main conclusion from the research carried out during the first year of DOD funding is that PKC ϵ may play a role in the tumorigenicity of prostate cancer cells. PKC ϵ may be relevant in proliferation, adhesion, anchorage-dependent and anchorage-independent cell growth. We will continue with our studies to address the role of PKC ϵ in the control of relevant signal transduction pathways. It is conceivable that PKC ϵ plays a role in modulating pathways implicated in cell death. We will continue with our studies to address the role of PKC ϵ in prostate cancer cell metastatic dissemination. We would like to establish proof-of-principle that pharmacological inhibition of PKC ϵ could represent a potential approach for prostate cancer therapeutics.

REPORTABLE OUTCOMES

None

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APPENDICES

FIGURE LEGENDS:

Figure 1: Generation of PKC ϵ depleted prostate cancer cell lines: DU145 (upper panel), PC3-ML (middle panel) and PC3 (bottom panel) cells were infected with shRNA lentiviruses for PKC ϵ (MISSION shRNA Lentiviral Transduction particles, #SHCLNV Clone ID TRCN844/845/846/847/848; Sigma, St Louis, MO, USA,) followed by selection with puromycin (0.3 μ g/ml). MISSION non-target shRNA Lentiviral Transduction particles (Sigma, SHC0016V) were used as control (NTC). Expression of PKC ϵ was analyzed by western blot using an anti PKC ϵ antibody (Santa Cruz, Santa Cruz, CA# sc-214). Vinculin (Sigma-Aldrich, St Louis MO, # V-9131) was used as loading control.

Figure 2: PKC ϵ is required for PC3-ML tumor growth in athymic nude mice. (a) PC3, DU145 and PC3-ML cells expressing shRNA control (NTC) or PKC ϵ (#844 and #848) at 80% confluency were resuspended in PBS, and then 0.1 ml containing showed number of cells were injected subcutaneously into the flank of male athymic nude-Foxn1^{nu} mice (Harlan Laboratories, Indianapolis, IN, USA). The width and length of tumors were measured with a caliper at different times, and tumor cross section area was calculated as $Vol = \pi \times w/2 \times L/2$. Data are expressed as mean. ($n=3$). (b) PC3, and PC3-ML cells expressing shRNA control (NTC) or PKC ϵ (#844 and #848) at 80% confluency were resuspended in PBS, and then 0.1 ml containing showed number of cells were injected subcutaneously into the flank of male athymic nude-Foxn1^{nu} mice. The width and length of tumors were measured with a caliper at different times, and tumor cross section area was calculated as $Vol = \pi \times w/2 \times L/2$. Data are expressed as mean. ($n=10$). (c) Representative pictures of mice showing tumors generated by NTC (red arrow) or PKC ϵ depleted cell (blue arrow) after 24 days. Tumors were removed and processed for immunohistochemistry (TUNEL), 15 days post inoculation.

Figure 3: In vitro characterization of PKC ϵ depleted PC3-ML cells: PC3-ML Parental cell (Parental), cells expressing shRNA control (NTC) or PKC ϵ (#844 and #848) at 80% confluency were synchronized to G0 and seeded in medium supplemented with 10% serum. (a) After 15, 30, 60 or 120 min attached (=viable) cells were quantified by Crystal violet staining. (b) After 48h attached (=viable) cells were quantified by Crystal violet staining.

Figure 4: ROS accumulation upon H₂O₂ treatment is higher in PKC ϵ depleted cells. PC3-ML Parental cells (P), cells expressing shRNA control (NTC) or PKC ϵ (#844 and #848) at 80% confluency maintained in the presence of serum were treated with H₂O₂ (a) Graph shows percentage of adherent cells (=viable) after 0.1 mM treatment of H₂O₂ for 4h. (b) Cells were incubated with DCFH 1 mM (Sigma) and treated with H₂O₂ 0.5 mM for 15 min. Representative pictures of analysis of DCFH-positive cells using an inverted fluorescence microscope.

Figure 5: PKC ϵ is required for the growth of PC3-ML cells. (a) To evaluate anchorage-independent growth, 3×10^3 cells were plated in 0.35% agar over a 0.5% agar layer. After 10 days, the plates were stained with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). (b) PC3-ML cells parental cell (P), cells expressing shRNA control (CTRL) or PKC ϵ (#844 and #848) at 80% confluency were synchronized and

plated in 100 mm plates (100 cells/plate). Medium was replaced twice a week, and after 15 days colonies were stained with 0.7% methylene blue in 50% ethanol.

Figure 6: Generation of PKC ϵ overexpressed prostate cell lines: (a) RWPE-1 cells were infected with ViraPower lentiviruses containing a Human PKC ϵ construct cloned into pLenti6/V-5 DESTRWPE-1. Expression of PKC ϵ was analyzed by western blot using an anti PKC ϵ antibody (Santa Cruz # sc-214). Vinculin was used as control. (b) RWPE-1 and RWPE-1-PKC ϵ cells at 80% confluency were synchronized to G0 and seeded in medium supplemented with 10% serum. After 48h the attached (=viable) cells were quantified by Crystal Violet staining.

Figure 7: PKC ϵ is required for migration and invasion in PC3-ML and PC3 cells. PC3 or PC3-ML parental cells(P), cells expressing shRNA control (NTC) or PKC ϵ (#844 and #848) at 80% confluency were synchronized and 2×10^5 were seeded in the upper compartment of Boyden chamber, and FBS was used as chemoattractant. (a) 12 or 8 μ m polycarbonate membrane for PC3 or PC3-ML respectively were used to separate both chambers. (b) 2×10^5 PC3-ML cells were seeded in upper compartment 12 μ m polycarbonate membrane coated with Matrigel was used to separate both chambers.

Figure 8: PKC ϵ -depletion does not affect activity of proteolytic enzymes in basal conditions: (a) Equal amount of volumes of conditioned medium (CM) of PC3-ML parental cells (parental), cells expressing shRNA control (CTRL) or PKC ϵ (#844 and #848) at 80% confluency were loaded in gelatin-impregnated gel (Invitrogen, Grand Island, NY; # EC6175BOX) and subject to gelatin zymography experiment following the manufacturer's protocol. *Left panel:* assessment of MMP-2 and MMP-9 activity using gelatin zymography. *Right panel:* assessment of MMP-2 and MMP-9 activity after heat-denaturation for 5 min at 90°C using gelatin zymography (b) *Righ panel* Equal amount of volumes of conditioned medium were loaded and subject to Comassie blue staining after electrophoresis to determined amounts of proteins in CM. *Left panel:* Expression of PKC ϵ of PC3-ML was analyzed by western blot using an anti PKC ϵ antibody (Santa Cruz; #SC-214). Vinculin (Sigma-Aldrich, # V-9131) was used as control.

Figura 1

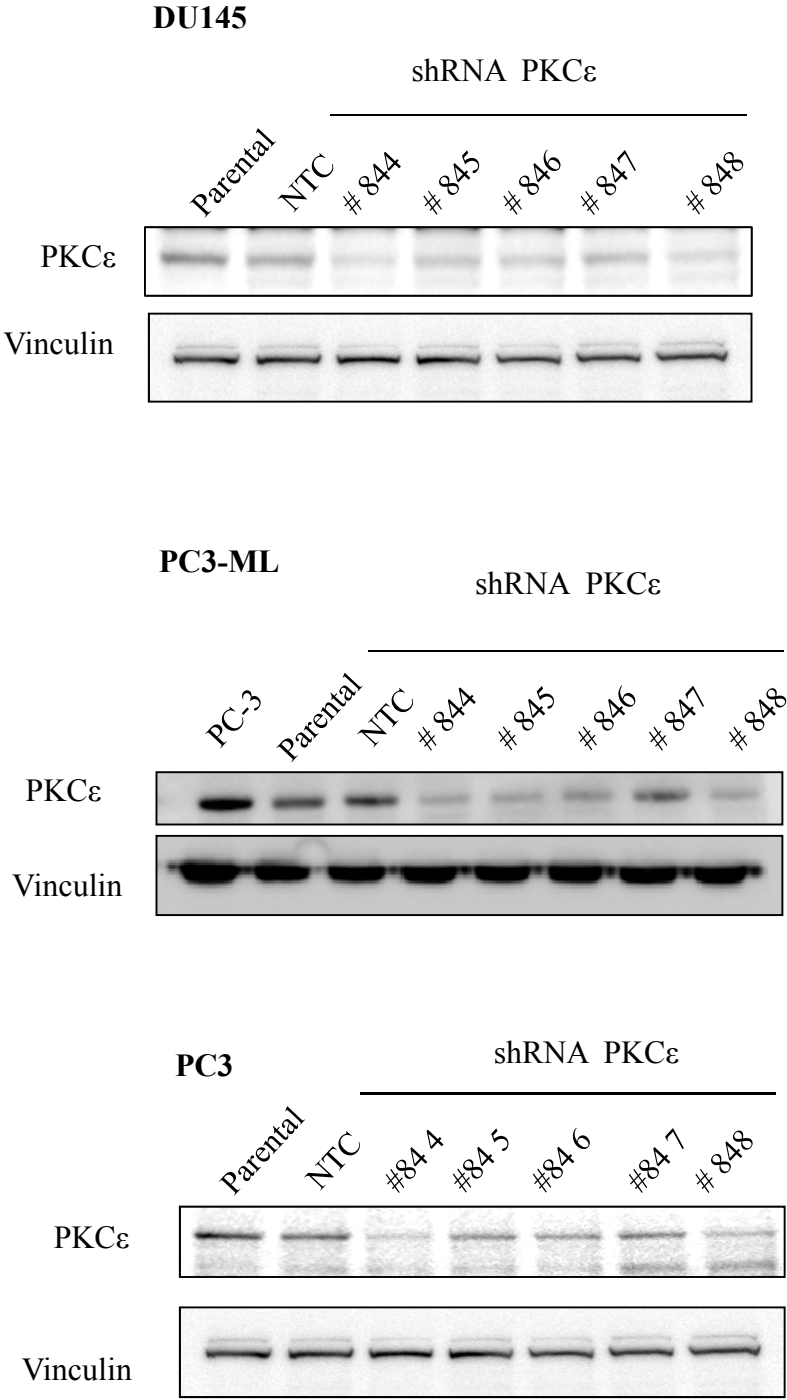
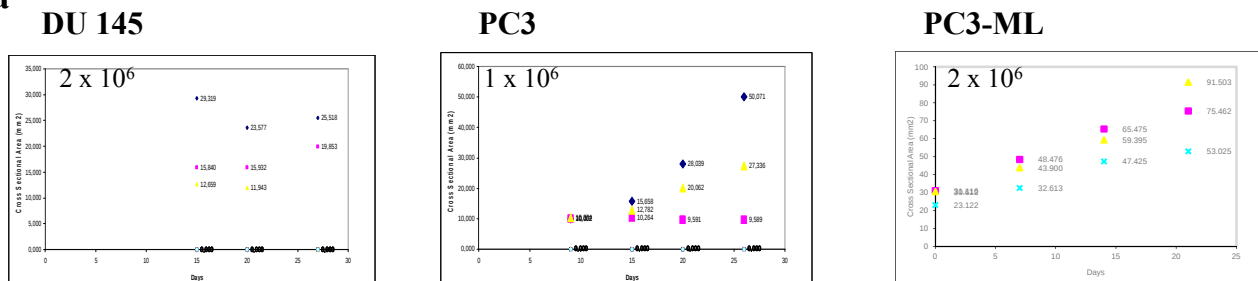
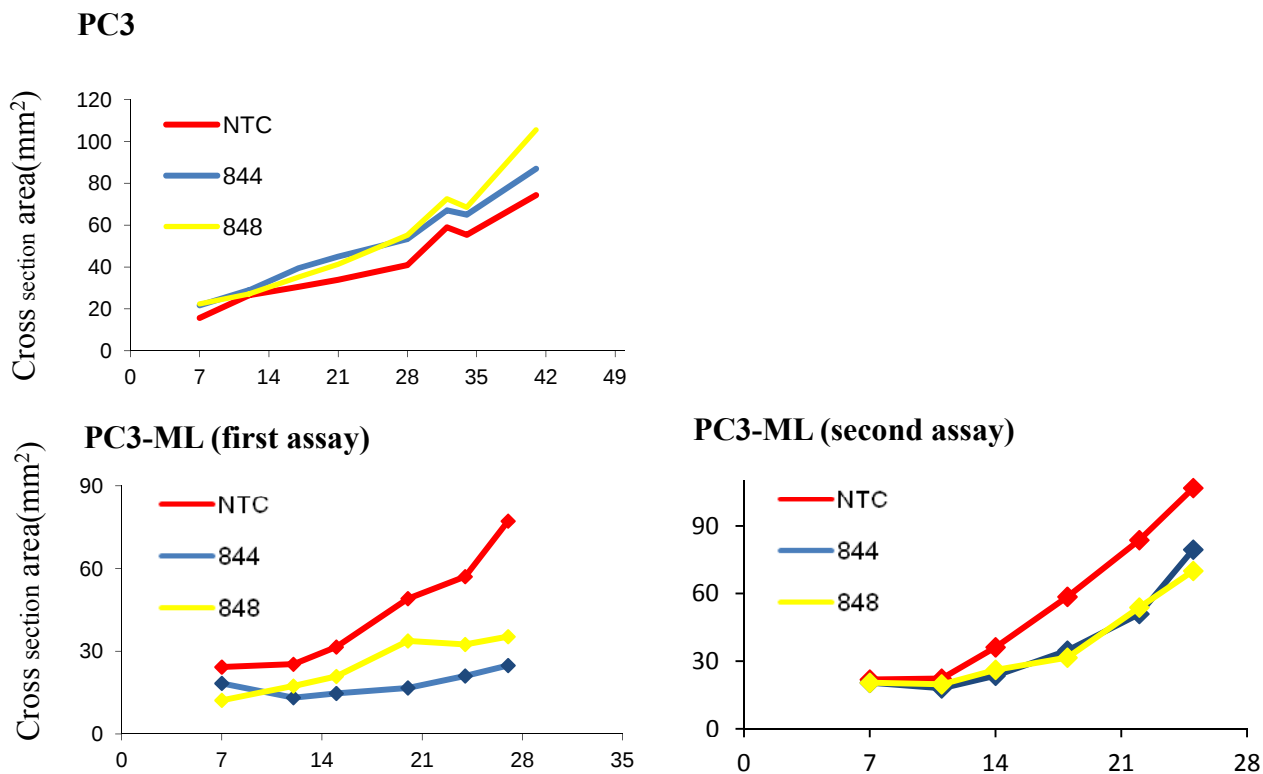


Figura 2

a



b



c

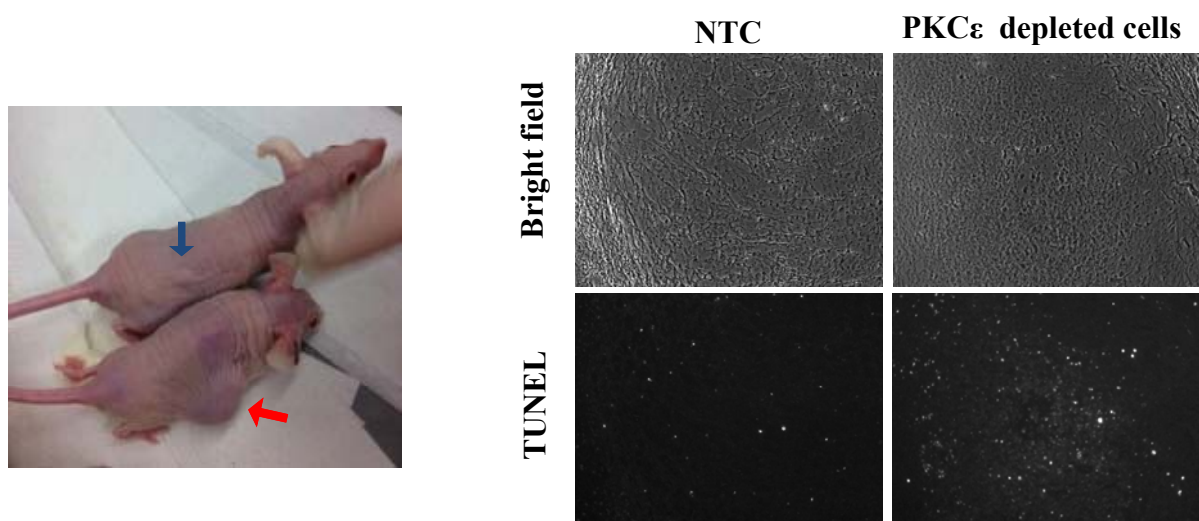
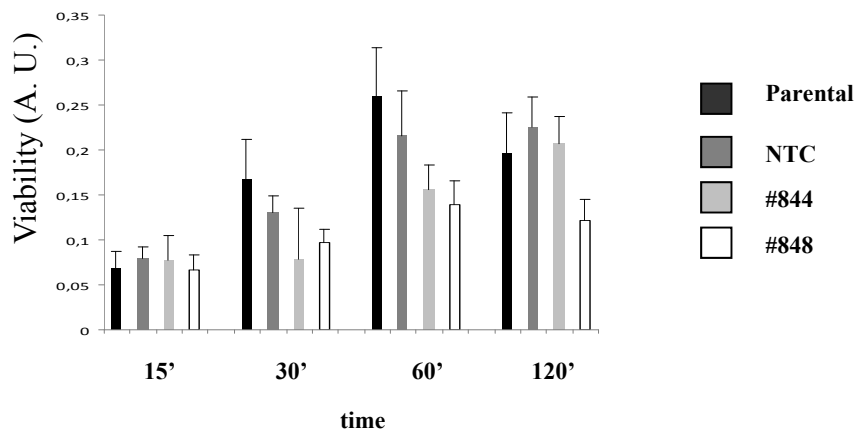


Figura 3

a

Adhesion Assay (PC3-ML)



b

Proliferation by CV staining PC3-ML

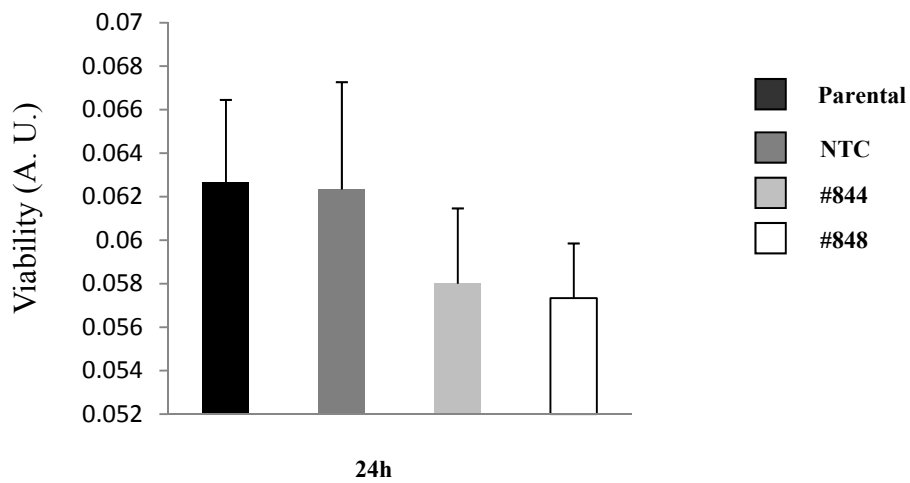
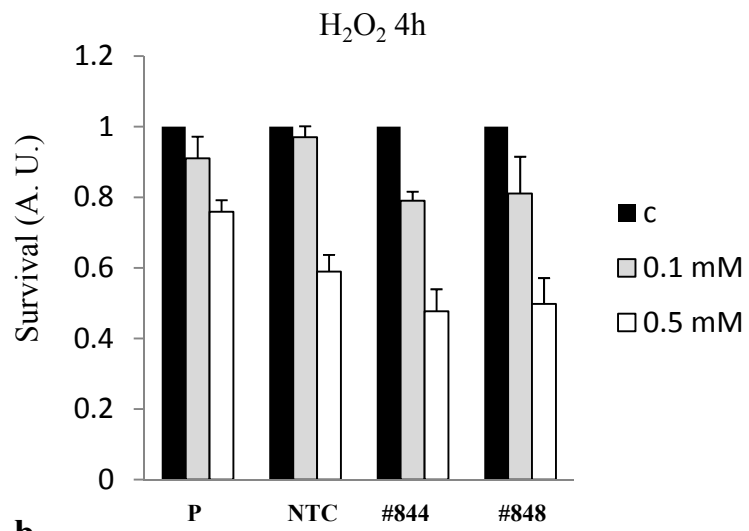


Figura 4

a



b

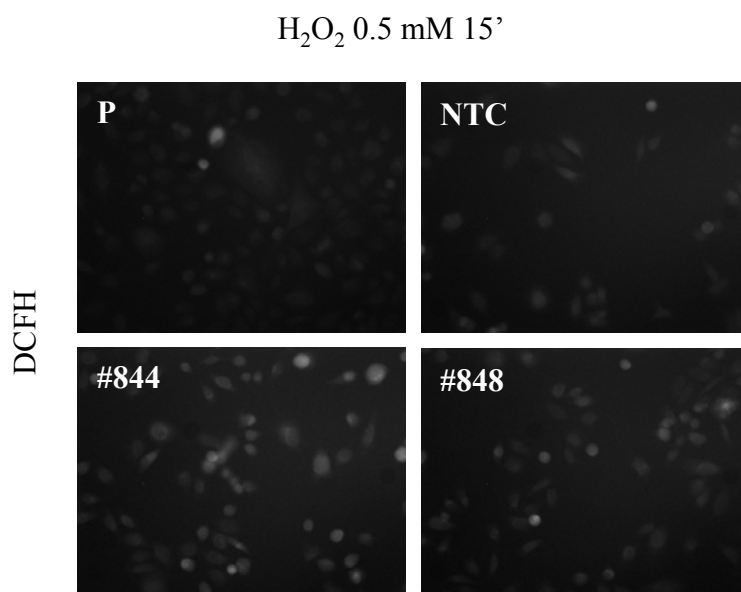
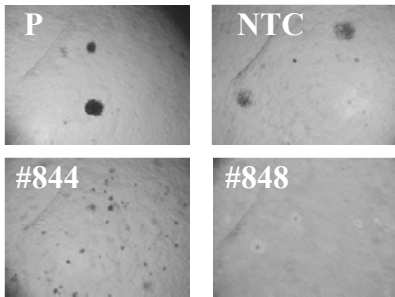


Figura 5

a

Anchorage independent assay



b

Anchorage dependent assay

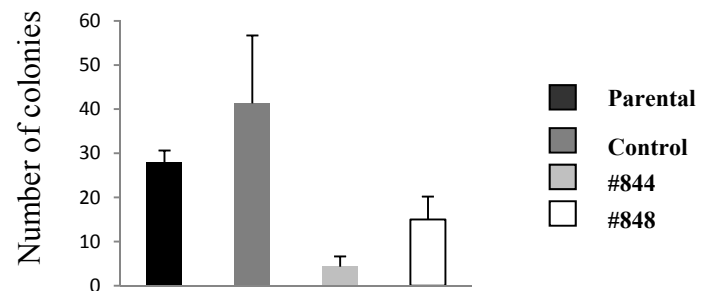
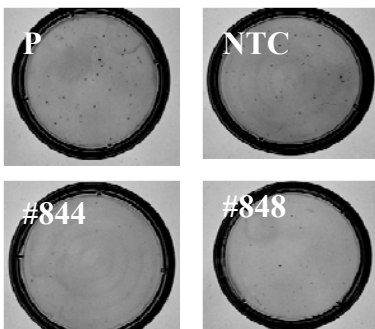
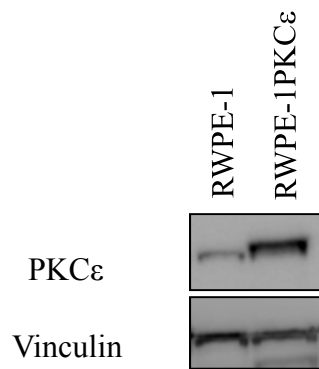


Figura 6

a



b

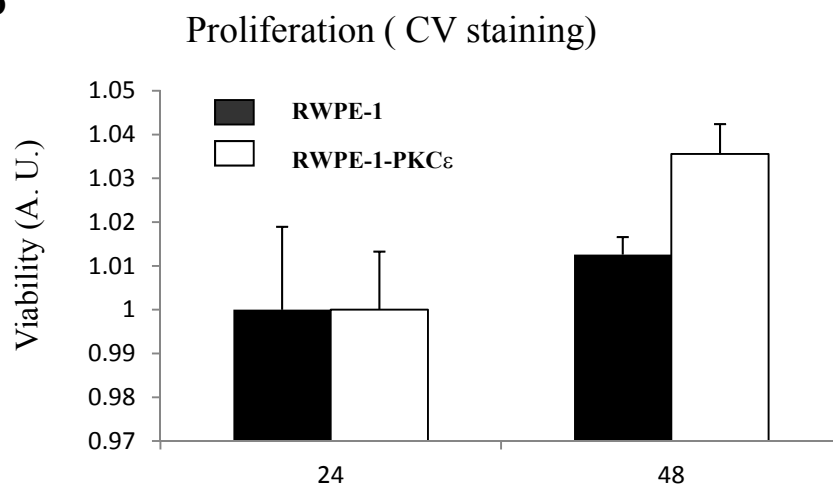
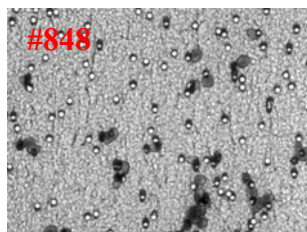
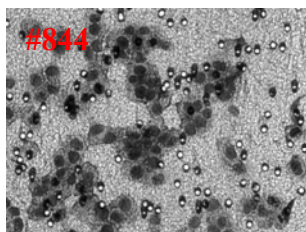
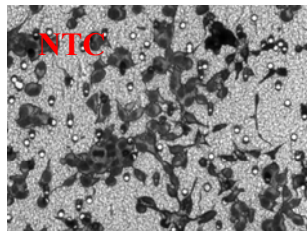
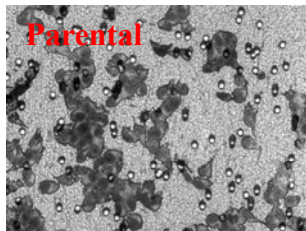


Figura 7

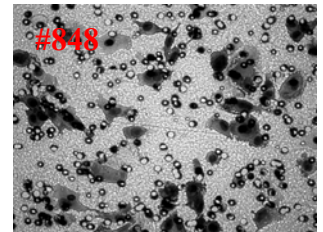
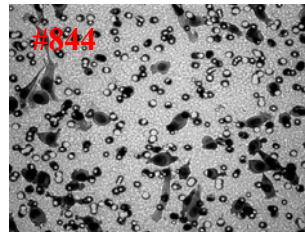
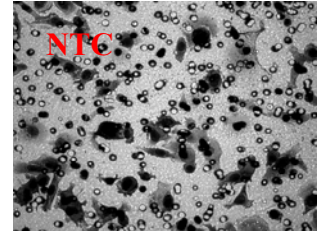
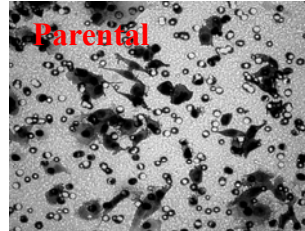
a

Migration assay:

PC3



PC3-ML



b

Invasion assay:

PC3-ML

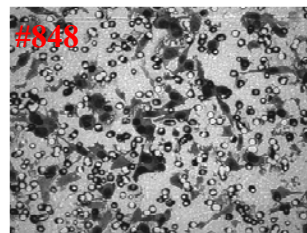
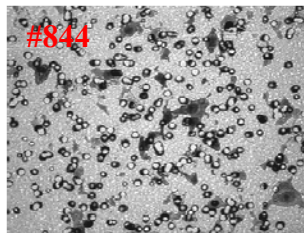
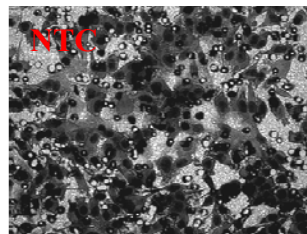
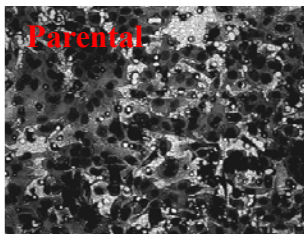
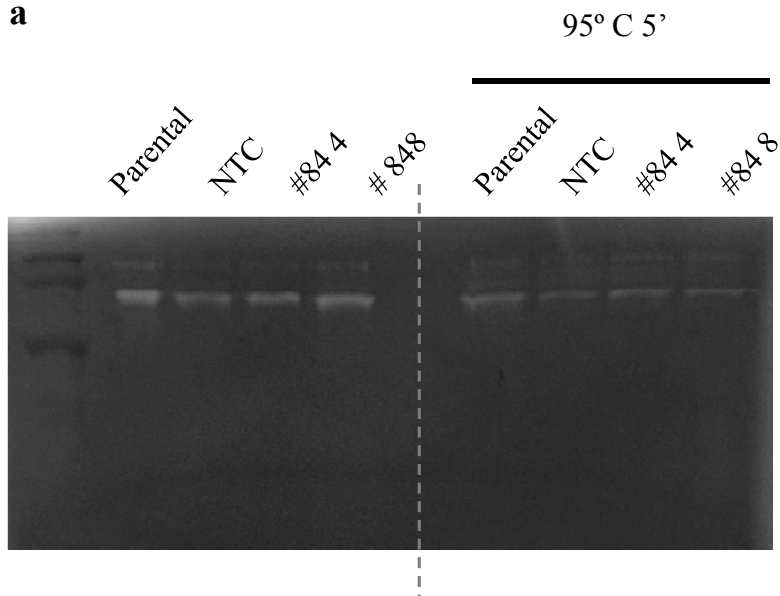


Figura 8

a



b

