GEM Cell Line Establishment, Characterization, and Analysis

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Abstract

The use of *in-vitro* cell lines in conjunction with i*n-vivo* mouse models is a common practice in the study of cancer. Culturing cells with the same genotypes as the specific strains of mice being used in studies can be very important and useful in supplementing the research and findings *in-vivo*. The purpose of this particular study was to develop, characterize, and analyze different cell lines in order to utilize them in conjunction with mice in the lab. We have successfully established two cell lines from two different mouse models. We determined that the two different genetic strains of mice generate two different types of cells when grown in culture that accurately reflect the source strains. The purpose of this project was to adapt a protocol meant for establishing human breast tumors to mouse prostate models, and then to test if the correct cells had been isolated for future use. Western blotting and other testing verified the correct cell type, therefore this project succeeding in developing a method for establishing more cell lines in the future.

Introduction

The United States reported approximately 220,000 new cases and 32,000 deaths due to prostate cancer in 2010 [1]. Prostate cancer is the most commonly diagnosed cancer in men and is the second highest source of cancer-related deaths in men in the United States. Although men diagnosed with localized prostate cancer have a 5-year survival rate of nearly 100%, men with advanced prostate cancer face a much more difficult challenge [1]. As a result, it is important to develop novel treatments for patients with advanced prostate cancer.

For many years, research in the Abate-Shen lab has focused on generating a series of genetically engineered mouse (GEM) models to evaluate drug therapies for prostate cancer. This project is part of a larger one aimed at evaluating drugs targeting the androgen receptor signaling pathways that are often used by prostate cancer cells as positive feedback loops promoting their growth and metastasis [2] [3]. This project takes advantage of the GEM models that are based on perturbations of molecular pathways that are frequently deregulated in human prostate cancer.

No model is perfect, and every experimental system has its own limitations. Mice breed relatively quickly and to an extent can be used to reflect human disease, but it can be expensive and very time consuming to develop strains with the necessary genotype in large enough quantities. Additionally, it takes 6-18 months for a mouse to develop prostate tumors. To choose a GEM strain to test a drug in terms of response and resistance, it would be beneficial to know which strains are susceptible and respond most effectively to treatment and which strains do not.

Drug screening in cell lines attempts to address this problem by creating a more efficient and accurate process of deciding which drugs to test on which GEM models. With a functioning bank of cell lines representative of each of the different strains held in the lab, any new drug can be tested on all or a selected few representative cell lines at any given point of time. This will reduce turnaround times compared to mouse models.

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In vitro models are, however, not without their limitations. There is always the fact that cells are dependent on their micro-environment, and drug response can be different in these models. Therefore it can sometimes be very challenging to extrapolate from the results of *in vitro* work back to the biology of the whole organism.

However, there are several additional reasons to develop mouse cell lines. A comprehensive collection of developed cell lines gives you the capability to ask questions and come up with answers quickly. Using shRNA, it is simple and quick in culture to knock out the expression of a certain gene of interest, and observe the effects of that on the growth of cancer cells. There is also the possibility of using the cell lines for injection into nude mice for xenograft studies. This would give an idea of how a tumor, given certain genetic alterations from the gene knockouts performed, would grow *in vivo*, albeit in a mouse without an immune system. Having and being able to manipulate cell lines reflecting our mouse models opens up many interesting experimental possibilities.

An understanding of the inducible nature of these mouse models is important for understanding this paper. *Nkx3.1* is a prostate-specific homeobox gene whose haploinsuffiency or loss predisposes to prostate cancer in humans as well as in mouse models. It codes for a recombinase that, when activated, cuts out other segments of DNA in specific places. It can either cut out stop sequences, promoting oncogene activation, or entire tumor supressors, promoting unchecked cancer growth. These alterations are reflected in the mouse models, and the goal of this research is to develop strains that maintain these genetic characteristics. This project is novel in that no other cell lines exist with these exact modifications, and that now we have a repeatable process for establishing them. The methods for establishing the cell lines as well as making sure they were the right ones follows, as well as a discussion of their implications.

Methods

GEM Models

The genetically engineered *Nkx3.1*$^{CreERT2/+;}$ *Pten*$^{flox/flox}$*; Kras*$^{LSL/+}$ mice (NPK mice) developed in the lab as well as *Nkx3.1*$^{CreERT2/+;}$ *Pten*$^{flox/flox}$*; Hi-Myc* (NPM mice) were used in this study. The *Nkx3.1* ^{CreERT2} allele simultaneously inactivates *Nkx3.1* and drives tamoxifen-dependent Cre-mediated recombination in adult prostate epithelium. Both strains have the *Nkx3.1*$^{CreERT2/+}$ allele, which is heterozygous for *Nkx3.1* and expresses *Cre-ERT2* under the control of the *Nkx3.1* promoter. These mouse models also contain a conditional allele for *Pten* (*Ptenflox/flox*) having loxP sites flanking exon 5. The NPK mice have a lox-stop-lox *Kras* allele expressing an inducible *Kras*^{G12D} allele (Figure A). The NPM mice express a transgene coding for the human *c-Myc* specifically in the prostate (Figure 1B). For induction of Cre activity, Tamoxifen is delivered by oral gavage (100mg/kg) for 4 consecutive days, to mice at 3 months of age. The inducible Cre allele also allows us to target gene deletion in the prostate epithelium of adult mice.

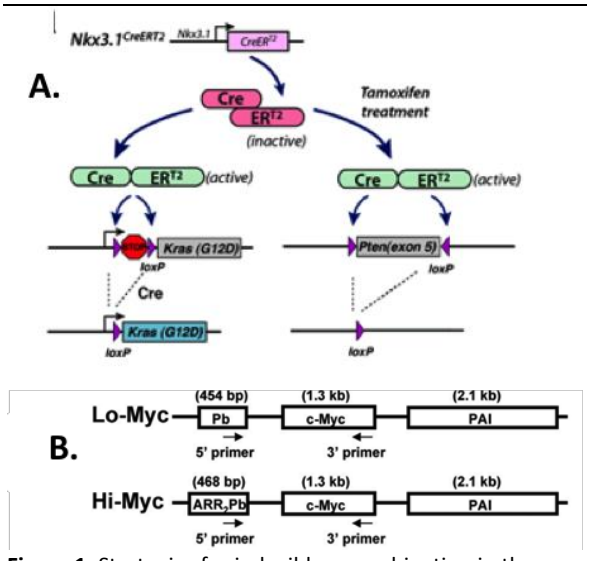

Figure 1: Strategies for inducible recombination in the prostate **[3]**

A. Diagram illustrating NPK mouse recombination

B. Diagram illustrating NPM mouse recombination

Cell Line Establishment

We adapted the protocol for culturing breast tumor cells from [4] to create our own prostate cancer cell line cultures. The steps, illustrated below, were as follows: 1) Prostate tumors were obtained from the NPK and NPM mouse models as well as metastases from lung and liver from the NPK mice. Each successful culture came from one mouse, but three NPK and three NPM mice were used, one final culture coming from each group. Tissues were harvested in DMEM media supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 10mM Hepes, 10ng/ml cholera toxin, 0.5µg/ml hydrocortisone, 5µg/ml insulin, and 5ng/ml epidermal growth factor (EGF). 2) The tissues were subsequently minced into small pieces. 3) The pieces were differentially centrifuged, and 4) placed into three different cultures labeled "organoid," "epithelial," and "mesenchymal" based on differential centrifugation (Figure 2). Cells were put onto primary culture plates with the supplemented DMEM media. 5) After several passages, the cells were then transferred to regular plates, and grown on RPMI media supplemented with 10% FBS. 6) Once the cells were homogenous in morphology and could survive 2-3 passes, the cells were considered "established." and frozen.

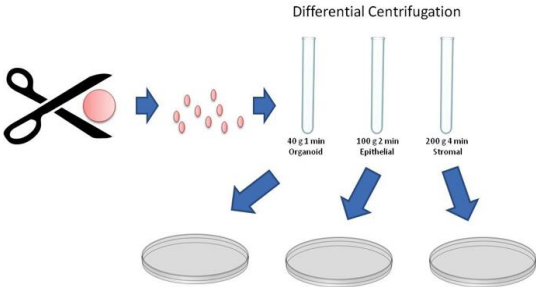

Figure 2: Schematic illustration of the cell separation procedure with the representative phase

Protein	Marker
Actin	Expressed in all cells, used to normalize western
Androgen Receptor	Expressed in prostate cells
Cytokeratin 5	Basal marker, expressed in epithelial cells of basal origin
Cytokeratin 8	Luminal Marker, expressed in epithelial cells of luminal origin
E-Cadherin	Epithelial Cell Marker
Phospho AKT and total Akt	Evidence of Mtor activation, consequence of Pten deletion
Phospho ERK and total Erk	Evidence of KRAS activation
Phospho S6 and total S6	Evidence of Mtor activation, consequence of Pten deletion
Vimentin	Expressed in stromal cells

Table 1: Description of the marker used to characterize the cell lines.

Cell Line Characterization

Cell lines were taken for protein quantification once they were established and 20 - 70 percent confluent. Protein was quantified using the Bradford method and 15 micrograms of proteins per gel lane was used. The table below describes each marker tested for and the reasoning behind it (Table 1).

Proliferation Rate

The proliferation rate of cells was also measured to see the relative rates of growth. Cells were plated at 50,000 cells per well for NPK cells and 100000 cells per well for NPM cells in 6-well plates in RPMI media with 10% FBS and counted every day for five consecutive days

Results

Cell Line Establishment

NPM and NPK Cell lines were successfully established in culture from the protocol outlined above. Five different strains were established from the NPM and NPK tumors: NPK Primary Prostate tumor (NPK-Pt), NPK lung metastasis (NPK-LM), NPK hepatic metastasis (NPK-HM), NPM primary tumor (organoid, NPM-Pt1), NPM primary tumor (epithelial, NPM-Pt2) Figure 1. The difference between the organoid and epithelial cells of origin for the NPM cells is that they were taken from different stages of the differential centrifugation process and grown separately (Figure 3).

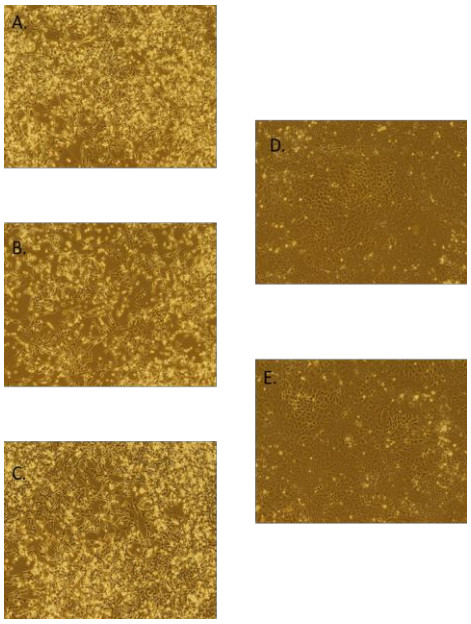

Figure 3: Pictures of the different cell lines, at 20x magnification. **A.** NPK-Pt cells in culture, **B.** NPK-LM cells in culture, **C.** NPK-HM cells in culture, **D.** NPM-Pt1 cells in culture, **E.** NPM-Pt2 cells in culture

Proliferation Rate

The doubling times of the NPK Pt, LM, and HM cells were found to be around 17 hours. The doubling time for the NPM cells was found to be 27 hours (Figure 4).

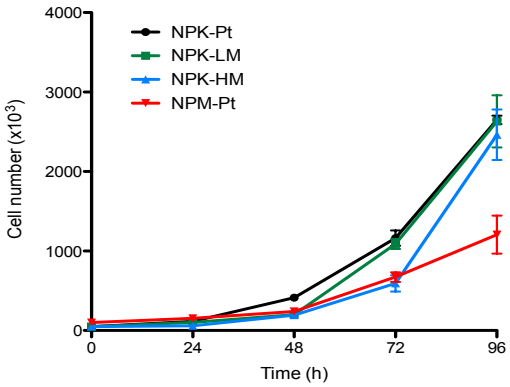

Figure 4: Growth curves of NPK-Pt, NPK-LM, NPK-HM, and NPM cells.

Western blot

Western blotting of five samples (NPK-Pt, NPK-LM, NPK-HM, NPM-1 and NPM-2) was conducted. The NPK cells all show high Cytokeratin 8 (CK 8), as well as expression of Phospho- S6 and Phospho- AKT. They do not appear to express CK5 at high levels and their E-Cadherin expression is not very high especially compared to the NPM cells. All NPM cells show high expression of CK 8 and E-Cadherin as well as expression of Phospho-AKT and Phospho-S6. NPM cells do not express Cytokeratin 5 at high levels (Figure 5).

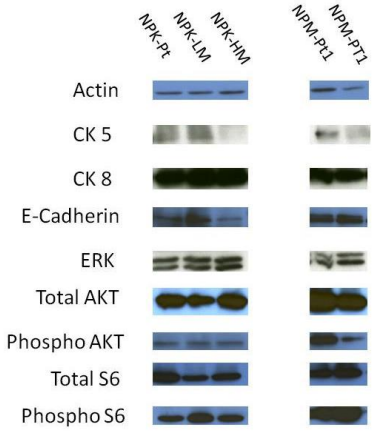

Figure 5: Western blot analyses using total protein extracts prepared from the different cell lines, NPK-Pt, NPK-LM, NPK-HM, and NPM cells.

Discussion

In the Speirs et al. paper, they experienced a rate of success with breast tissue of around 30% with the full establishment of cell lines. We experienced a very similar success rate of around 33% from our own attempts. It is important that we now have a protocol for setting up cell lines from our mouse models, and my future research will focus on setting up lines for all the models as well as characterizing them in the ways described previously.

After the cell lines were established, it became clear that their morphologies were

quite different. The cell lines from the NPM clones appeared to be more epithelial in nature, as shown below when compared to breast epithelial cells from [5] and the *Kras* tumors cells look more fibroblastic. Previously it was shown in the lab that *Kras* tumors contain EMT (epithelial mesenchymal transition) cells and western blot supports this (Figure 6) [3].

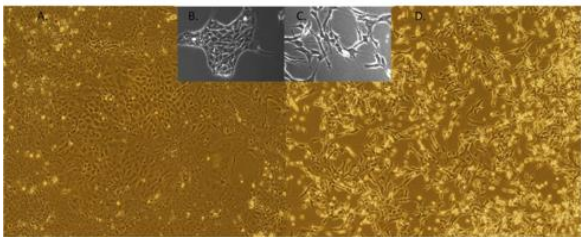

Figure 6: Comparison of Experimental cells with known epithelial cells and cells undergoing EMT.
A. NPM-Pt1 cells
B. Breast epithelial cells
C. Breast cells undergoing EMT
D. NPK-Pt cells

The graph showing cell proliferation reflects the *in vivo* findings that *Kras* mice have especially aggressive tumors that grow very quickly, while the NPM mice have slower growing tumors [7].

The western blots helped confirm that both the NPM cells were indeed epithelial cells with PTEN deletion. The presence of E-Cadherin in all the cells signifies that they are at least partly epithelial in nature, and the fact that it is expressed to higher levels in the NPM cells is consistent with their more epithelial morphology. As NPK cells did not express E-Cadherin as strongly, it can be said that they are not as epithelial in nature, supporting the idea that they are undergoing EMT. The fact that they all express phosphor-S6 as well as phosphor-Akt indicates that the Akt/Mtor pathway has been activated in response to Pten deletion. When prostate cancer cells take to culture, it has been shown that they become more luminal as opposed to basal in nature, and that occurred in these cells as well. CK8 (a luminal marker) was strongly expressed in all cells while CK 5 (a basal marker) was not as highly expressed [6].

The western blot for AR, vimentin, and phosphor-ERK will be performed as part of future research. AR would confirm the prostate nature of the cells, vimentin would indicate how mesenchymal they are in nature (which could confirm the suspicion that the Kras tumors are EMT in nature), and phosphor-ERK would show activation of Kras. In the future, Myc also will be tested to make sure that NPM cells express Myc.

Further research will include the establishment and characterization of other cell lines from others GEM models that show different genetic alterations, as well as drug screening, xenograft studies, and gene knockdown studies, as discussed in the introduction. This project provides a protocol and a basis for the future development and characterization of additional cell lines.

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