45

DEVELOPMENT OF CRISPR-CAS9 MEDIATED PROTEIN KNOCKOUT CELLS AS A TOOL AND RESOURCE FOR PROSTATIC RESEARCH

Mary M. Stangis[1,2,4,6,], Hannah N. Miles[1,3,6,*], Teresa T. Liu[1,5,6], William A. Ricke[1,4,6]*

George M. O'Brien Urology Research Center
Department of Urology
UNIVERSITY OF WISCONSIN
SCHOOL OF MEDICINE AND PUBLIC HEALTH

Department of Urology[1], Cancer Biology Training Program[2], Pharmaceutical Sciences Training Program[3], Carbone Comprehensive Cancer Center[4], K12 Urologic Research Career Development Program[5], George M. O'Brien Center of Research Excellence[6]: University of Wisconsin-Madison, Madison, WI *These authors contributed equally to this project

Cancer Biology Graduate Program · Li Research Group · CAIRIBU 2021 Annual Meeting · NIH National Institute of Diabetes and Digestive and Kidney Diseases

Introduction & Objectives

Since its discovery in 2012, CRISPR-cas9 has become a staple technique amongst molecular biologists. Using the cas9 endonuclease, precise DNA strand breaks can be introduced to allow for a wide variety of downstream applications including insertion of new DNA sequences, removal of sequences, and knockout (KO) of protein expression. Here, we introduce a method combining stable CRISPR-cas9 expression and transient puromycin resistance to allow for development of cell lines with multiple KO proteins.

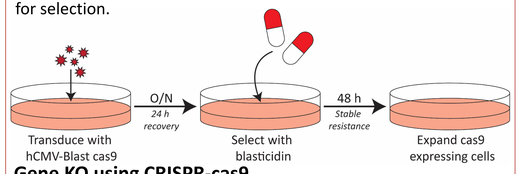
Materials and Methods

Cell Lines and Culture Conditions

For this project, we utilized a variety of prostate cell lines representing both normal and diseased tissue. For the purposes of this poster, we will be focusing mainly on our work with BPH-1 (benign prostate hyperplasia, epithelial) and BHPrS-1 (benign human prostate, stromal). Majority of cell lines utilized were cultured in RPMI-1640 + 5% FBS + 12.5% HEPES + 0.2% Normacin growth medium and maintained at 37 ˚C with 5% CO_2.

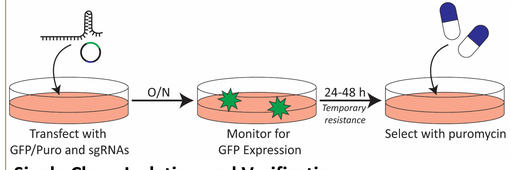
Lentiviral Transduction of Cas9 Endonuclease

Lentiviral particles containing the genes for the Cas9 endonuclease and resistance to blasticidin were purchased from Dharmacon (VCAS10124). Cells were seeded in triplicate wells of a 12-well plate and allowed to grow in complete growth medium overnight. Transduction was performed overnight in Opti-MEM supplemented with polybrene. The following morning, Opti-MEM was removed and replaced with complete growth medium. Cells were allowed to recover for 24 hours, and then treated with 80 µg/mL blasticidin for selection.



Gene KO using CRISPR-cas9

Cas9 expressing cell lines were expanded and prepared for transfection with sgRNAs for genes of interest (Edit-R sgRNA; also purchased from Dharmacon) by seeding into a 12-well plate for overnight growth. For transfection, we used 3 different sgRNA sets for our gene of interest and two different control sgRNAs: a non-targeting sgRNA for a negative control, and an sgRNA for DNMT3B. Control sgRNAs were used at 75 nM, sgRNAs for gene of interest at 50, 75, and 100 nM. One well received no sgRNA and was used as a naïve control. Cells were co-transfected with a plasmid for GFP and puromycin resistance at 1 µg/ well. Transfection mix was prepared using the appropriate amount of plasmid and sgRNA, as well as Dharmafect Duo transfection agent, in Opti-MEM and applied to cells overnight. Opti-MEM was then removed and replaced with complete growth medium, and cells were observed for GFP using an ECHO fluorescence microscope at 24- and 48-hours post-transfection. Once GFP was observed, cells were treated with puromycin at 0.4 – 0.8 µg/mL for 48 hours, depending on cell line.



Single Clone Isolation and Verification

Following puromycin treatment media was refreshed, and cells were allowed to recover to confluence. Cells were then plated at a density of 10 cells/mL into 5 replicate 96-well plates to isolate monoclonal cultures. Gene KO was verified using qPCR, Immunocytochemistry (ICC), and Western Blot.

Results

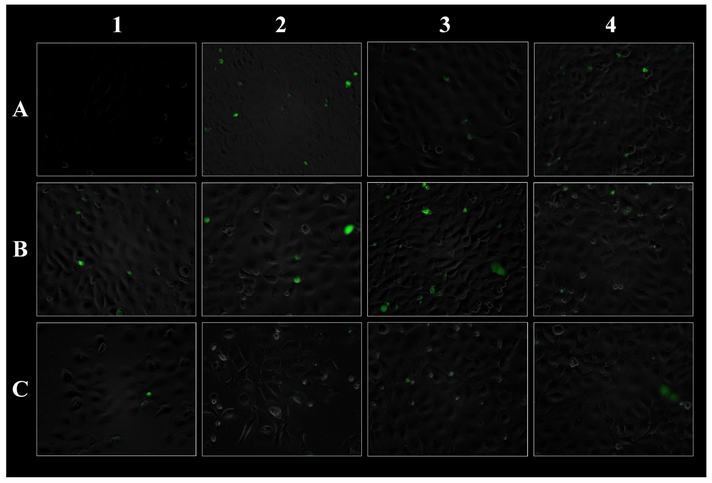
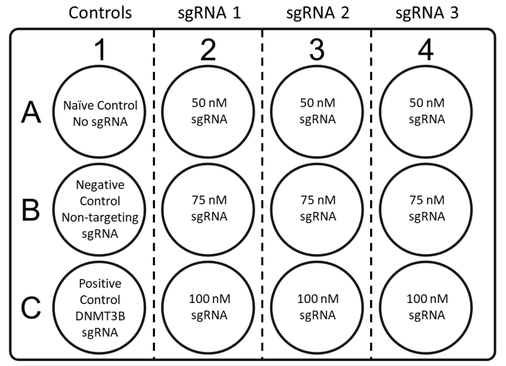


Figure 1: GFP expression in BPH-1 cells, 48 hours post-transfection with sgRNAs and GFP/Puromycin resistance plasmid. Cells in **A1** were not transfected, represent a naïve control, and show no GFP expression as expected. Plate layout below.



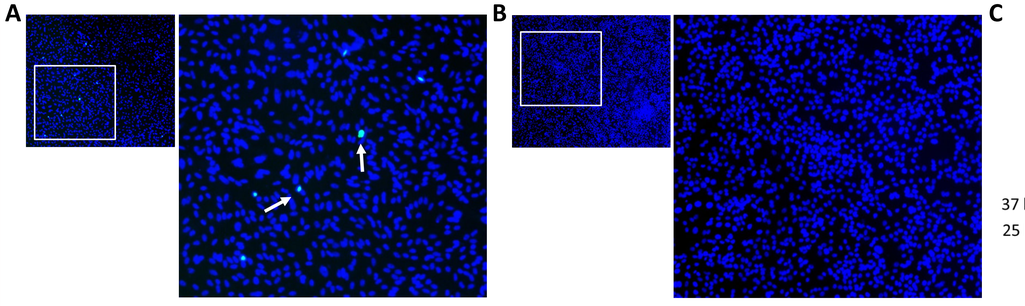


Figure 2: Immunocytochemistry (ICC) for FGF-5, DAPI in PC3 cells. **A.** PC3 cells with FGF-5 expression notated with white arrows. **B.** CRISPR-KO PC3 clonal population lacking FGF-5 expression. **C.** Western Blot analysis of CRISPR modified PC3 clonal populations was performed to confirm initial ICC findings. FGF-5 has an approximate size of 29 kDa, suggesting that these clones are truly FGF-5 KO.

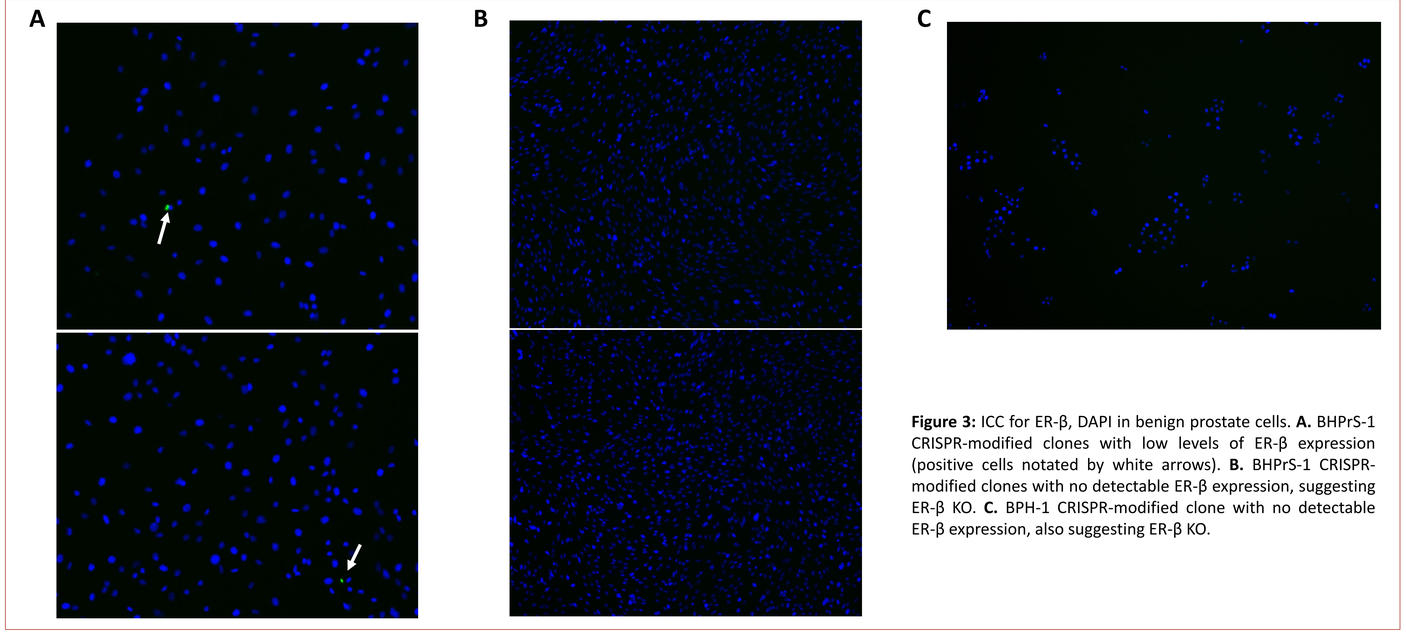


Figure 3: ICC for ER-β, DAPI in benign prostate cells. **A.** BHPrS-1 CRISPR-modified clones with low levels of ER-β expression (positive cells notated by white arrows). **B.** BHPrS-1 CRISPR-modified clones with no detectable ER-β expression, suggesting ER-β KO. **C.** BPH-1 CRISPR-modified clone with no detectable ER-β expression, also suggesting ER-β KO.

Results (Cont.)

Cas9-expressing KO Cell Lines Currently Available

Protein of Interest	Cell Line	Cell Type
FGF-5	PC3	Human Prostate Cancer
	BCaP MT10	BCaP Model, Tumorigenic
	LNCaP	Human Prostate Cancer
ER-β	BHPrS1	Benign Human Prostate Stroma
	BPH-1	Benign Prostate Hyperplasia (Epithelial)
DDX3(X)	BCaP MT10	BCaP Model, Tumorigenic

Cell line currently in final stages of protocol. Additional cell lines such as C4-2B and remaining BCaP Model lines are at the stable cas9 expression stage.

Conclusions

Thus far we have shown that our proposed technique is able to produce stable KO of a protein of interest in both prostate cancer and benign prostate cells – and cells of both stromal and epithelial origin. We have successfully generated CRISPR-mediated KO cell lines for FGF-5 and ER-β at this time. Next, we plan to show that this novel technique will allow for easy generation of double, triple, and potentially quadruple KO cell line generation as the puromycin resistance conferred to the cells during transfection is transient.

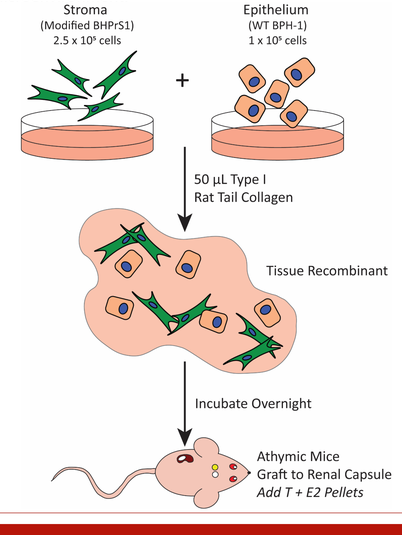
Future Applications

Cell Signaling Studies *In Vitro*

Cell lines generated using this technique could be used to study cell signaling pathways *in vitro* though use of the transwell culture system, and use of existing BHPrS1 and BPH-1 cell lines. By knocking out a receptor or ligand of interest in one population of cells, and co-culturing with the other via transwell, researchers could look at signaling downstream of the receptor or ligand targeted in the unmodified population of cells.

Recombinant Tissue Grafts for *In Vivo* Study

Cell lines generated using this technique could also be used to prepare recombinant tissue grafts using existing protocols and used to study the effect of the loss of the protein of interest *in vivo* in the microenvironment.



Acknowledgements

U54DK104310