

shRNA Phenotypic Screen To Identify Gene Targets Contributing to Androgen Independent Prostate Cancer Cell Growth

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Introduction

For patients presenting with disseminated prostate cancer, the tumor is typically dependent on androgen for growth and therefore responsive to therapies that take advantage of surgical and/or pharmacological depletion of circulating androgens. However, this type of therapeutic success is often temporary; the disease almost invariably recurs as a metastatic and androgen-independent tumor. In an effort to identify pathways whose inhibition complements androgen ablation, we screened a panel of shRNAs targeting 712 human kinases against LNCaP prostate cancer cells grown in the presence and absence of hormone. Lentivirally delivered shRNA enables a rapid method to produce stable transcript knockdown to conduct a loss of function genetic screen. Alterations in cell proliferation rates were determined after knockdown in the presence and absence of androgen. During the course of our study careful optimization of screening parameters including cell growth conditions, multiplicity of infection, puromycin selection, androgen treatment and cell viability measurements was performed. This study demonstrates the applicability of lentiviral based shRNA for developing and executing phenotypic screens for the identification of targets involved in a variety of biological processes, including regulation of prostate cancer cell growth by growth factor and androgen signalling demonstrated herein.

Material and Methods

All reagents and materials used were from Sigma except for methyltrienolone (R1881), which was obtained from PerkinElmer. LNCaP cell line was maintained in RPMI 1640 with no phenol red, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10mM HEPES, 1.0 mM sodium pyruvate, 10% heat inactivated FBS, and 1× penicillin / streptomycin solution, and used before passage 50 in all experiments.

Screen Materials and Methods

MISSION®LentiExpress Human Kinase Panels, one panel in the presence of R1881 and one in the absence of R1881, were used in triplicate per condition. Controls included, in four wells per plate, lentivirus with shRNA against the androgen receptor (NM_000044). Androgen receptor knockdown was stable under the experimental conditions of the assay (data not shown). Also included in four wells per plate each were MISSION pLKO.1-puro Control Transduction Particles and MISSION Non-Target shRNA Control Transduction Particles. LNCaP human prostate cancer cell line was reverse-transduced at 6,000 cells per well at an MOI of approximately 1 in the presence of 8 µg/mL polybrene (hexadimethrine bromide), then incubated with virus for approximately 18 hours in a 37 °C, 5% CO₂ incubator. Medium was changed and contained either complete medium with 50 pM R1881 or complete medium prepared with charcoal-stripped FBS (csFBS). Forty-eight hours post-transduction, all panel plates were placed under puromycin selection, and at 96 hours post-transduction, medium was changed to fresh complete containing puromycin. On day seven post-transduction, medium was changed with resazurin solution added at 10% of the medium volume to all plates. Plates were incubated as described for

three hours and fluorescence was read at an excitation of 560 nm and an emission of 590 nm. Data were analyzed by subtracting out background of complete medium plus resazurin, combining three biological replicates, and converting all transduced wells in both presence and absence of R1881 to a growth percentage as compared to MISSION pLKO.1-puro Control wells, which were set at 100%.

Results

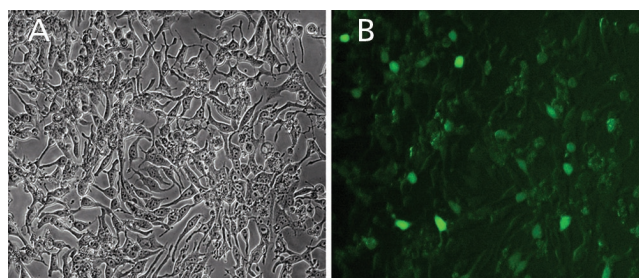


Figure 1: LNCaP 7 days post reverse transduction and four days under puromycin selection at an MOI of 1. Panel A shows cells surviving puromycin selection, and Panel B shows the same cells expressing GFP.

LNCaP Cell Viability Using Resazurin as a Function of Cell Number and Time (n=10)

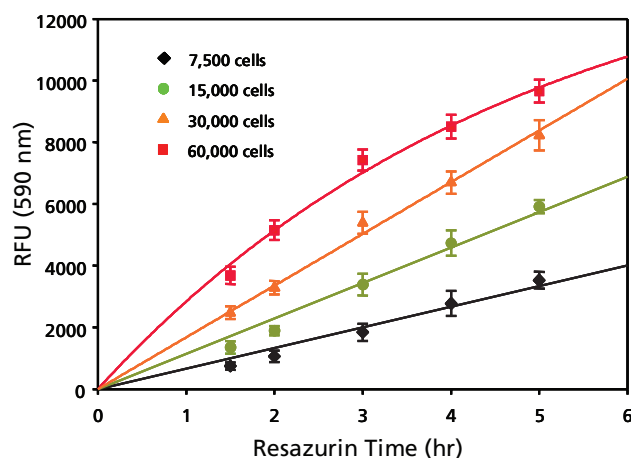


Figure 2: LNCaP cells were serially diluted in a 96 well plate, incubated overnight, then incubated for varying times with the viability dye resazurin demonstrating reproducibility and sensitivity.

Four shRNAs for the androgen receptor were compared and the construct that yielded the greatest reduction in androgen receptor expression was chosen as a control for use throughout the screen to show inhibition of growth in both plus R1881 and minus R1881 conditions (**Figure 3**).



Androgen Receptor Knockdown Normalized to Untreated Cells and Cyclophilin B (MOI 5)

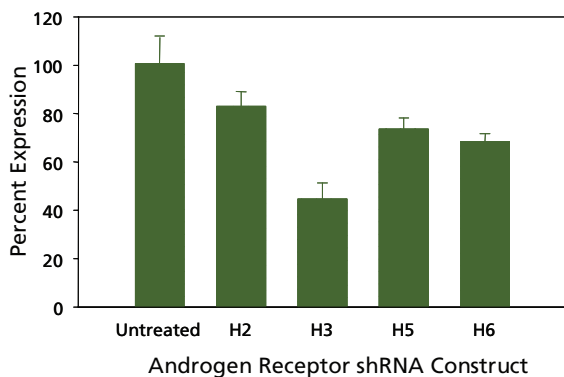


Figure 3: qRT PCR results comparing gene expression of four different shRNAs targeting androgen receptor in LNCaP cells.

Androgen receptor expression of clone H3 was reduced approximately 55%, which was enough repression to inhibit LNCaP growth but not enough to kill the cells during the course of the assay (**Figure 4**).

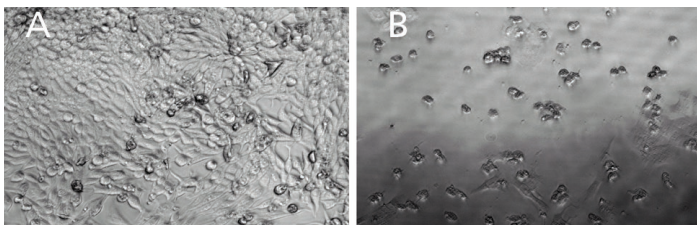


Figure 4: Panel A shows LNCaP cells transduced with MISSION Non-Target shRNA Control Transduction Particles and Panel B shows LNCaP cells transduced with lentiviral particles containing H3 shRNA for the androgen receptor.

Growth inhibition and induction as compared to pLKO.1-puro controls under both androgen and androgen-free conditions were found (**Figure 5**). For the purpose of analysis biological replicates were combined, and growth and inhibited phenotypes for both test conditions were converted to a percentage of the pLKO.1-puro controls, which were set to 100%.

shRNA Kinome Screen: LNCaP

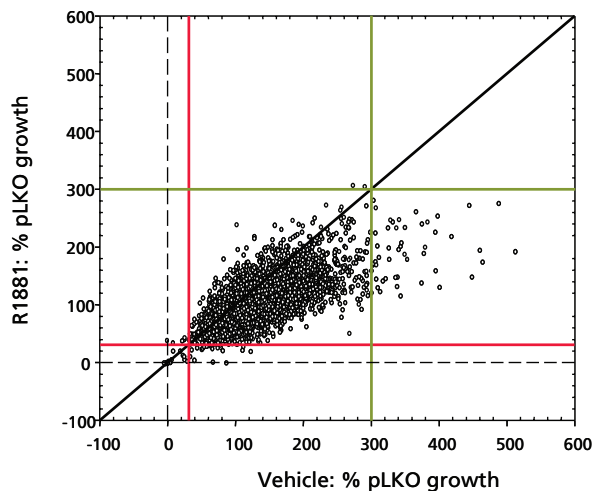


Figure 5: Graph showing all data points derived from screen in both the presence and absence of R1881. Dotted lines represent the zero axes, red lines define kinases that inhibited growth at 25% of the pLKO.1-puro controls, and green lines define kinases that increased growth three-fold over the pLKO.1-puro controls.

Discussion

Results from this shRNA screen identified multiple shRNA clones against the same gene targets that produced similar phenotypic changes. Specifically, targets such as MAP2K4, which had been previously shown to be involved in regulating androgen sensitivity, and other gene candidates that are involved in MAPK signalling or are part of the MAPK family, ERK signalling and Alpha/Beta Andrenergic signalling were identified. Furthermore, glycogen synthase kinase 3 beta, ribosomal protein S6 kinase (RPS6KA3), and p21 (CDKN1A)-activated kinase 6 (PAK6), all previously implicated in prostate cancer, inhibited growth in both androgen and androgen-free conditions. Several novel proteins were identified as well that may provide hints to the molecular mechanisms that lead to androgen independence in prostate cancer, or that may provide clues for therapeutics. These data provide a proof-of-concept that with optimized conditions and standardized practices, RNAi screens can be useful tools for mining cellular pathways for clues regarding disease states and for the development of therapeutics.