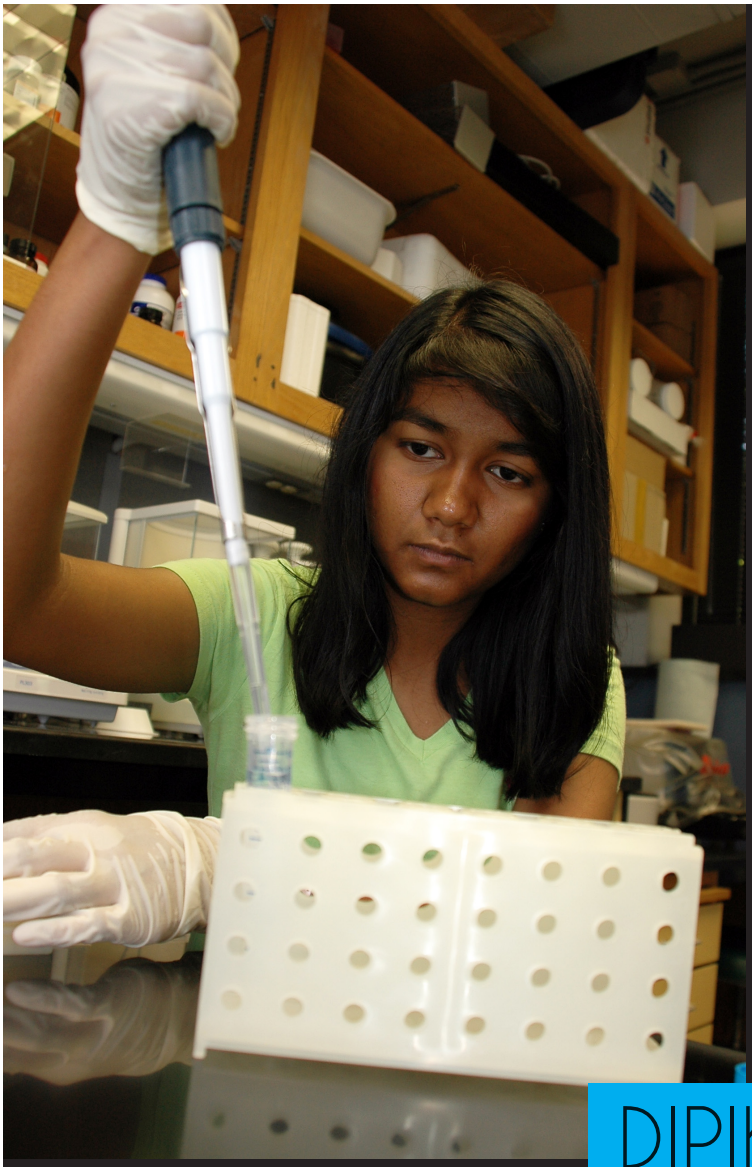


2010

RICHARD K. & RUTH S. LANE MEMORIAL SCHOLAR

DIPIKA MOHAN

BROKEN ARROW, OKLAHOMA

FRESHMAN, WASHINGTON UNIVERSITY

FINAL RESEARCH MANUSCRIPTS

OMRF

Investigating the Molecular Role of LDOC1, a Novel Cancer-Related Gene

Dipika Mohan

Broken Arrow, Oklahoma
Freshman,Washington University, St. Louis, Missouri

Mentor: Jonathan Wren, Ph.D.
Colleagues: Mikhail Dozmorov, Ph.D.

Oklahoma Medical Research Foundation, Arthiritis and Immunology Research Program

ABSTRACT

LDOC1 (leucine zipper, down-regulated in cancer 1) is a gene of unknown function, initially noted to be down-regulated in various human pancreatic and gastric cancer cell lines, though expressed ubiquitously in most normal tissues. It was additionally found to be up-regulated in breast cancer and other types of cancer. LDOC1 is poorly characterized—only three notable studies document its correlative behaviors.

GAMMA (global microarray meta-analysis), a data-mining program developed by Dr. Jonathan Wren, predicted LDOC1 would be associated with cell adhesion, the epithelial-mesenchymal transition, and cell migration, all cancer-related processes. We are empirically testing these hypotheses and hope that understanding its function may ultimately aid in prevention of cancer development and metastasis. We investigated LDOC1 expression in bladder cancer and prostate cancer cell lines so that we can identify those whereby we can manipulate the levels of LDOC1 and observe phenotypic effect. After isolating RNA and synthesizing cDNA from these cell lines, we used single-step qPCR to measure levels of LDOC1 expression. LDOC1 expression was then silenced using two siRNA techniques: ectopic transfection of Cy3-labeled siRNA complexes, and nucleofection of Cy3-labeled siRNA complexes into cells. We observed through microscopy the PC-3 cell lines as most easily transfected, and the nucleofection method as yielding the most LDOC1 expression silencing. We then performed a wound-healing assay on PC-3 cells testing the phenotypic effects of LDOC1 silencing. We hope to better understand both the specific molecular role of LDOC1 and its relation to cancerous processes.

INTRODUCTION

LDOC1 (leucine zipper, down-regulated in cancer 1) is a gene with no known molecular function, with only three studies of note published to date regarding observations and correlations. Though expressed ubiquitously in normal tissues, LDOC1 was initially noted to be down-regulated in various human pancreatic and gastric cancer cell lines1. LDOC1 expression was found to induce apoptosis in a manner enhanced by the MZF-1 (myeloid zinc finger 1) transcription factor2. It was suggested by a final study that LDOC1 inhibits NF-κB (nuclear factor kappa B)3, a transcription factor responsible for encouraging cell proliferation and protecting cells from apoptosis. Conversely, LDOC1 was also found to be up-regulated in breast cancer and other types of cancer—LDOC1 is also known as BCUR1 (breast cancer, up-regulated 1)—causing us to question what exactly the role of LDOC1 is to cancer.

LDOC1 was first identified as a gene of interest by GAMMA (global microarray meta-analysis)4, a data-mining program developed by Dr. Jonathan Wren and sustaining further modifications by Dr. Mikhail Dozmorov. The program evaluates microarray data obtained from a public repository, NCBI's Gene Expression Omnibus (GEO). Via an analysis of gene-gene co-expression behavior across different experimental conditions, GAMMA can predict gene phenotype. In accordance with published associations, it indicated that LDOC1 is implicated in pancreatic cancer and prostate cancer, as well as various other mechanisms. GAMMA also predicted that LDOC1 is involved in cell adhesion, the epithelial-mesenchymal transition, and cell migration, all testable, cancer-related processes. As a centralized, phenotypic starting point to investigating the poorly characterized function of LDOC1, GAMMA helped to pinpoint the fundamental methodological approach to take for the study by characterizing predicted associations. Following, Table 1 shows testable predictions provided by GAMMA, and Figure 1 serves to indicate LDOC1's very thorough presence in protein interactions and gene expression, providing further motivation to researching its unknown absolute function.

MATERIALS AND METHODS

Because of the unknown biological role of LDOC1 and the ability to pinpoint associations using GAMMA, the methodology of our study was designed so that we could effectively establish a phenotype for the gene's expression through silencing. Thus, our initial objective was to find a cell line with reasonable LDOC1 expression and effective silencing. We cultured various cell lines, quantified their LDOC1 expression, silenced LDOC1, and measured the extent of RNAi-mediated mRNA silencing. After these steps, we performed a wound-healing assay intended to phenotype LDOC1 by simulating a wound and allowing us to assess the observable characteristics of cell migration and cell adhesion in "healing" this wound. These steps, explained in more detail following, helped to define our experiment as fundamentally substantial in understanding more specifically the functionality of LDOC1.

In researching the role of LDOC1 in cancerous processes, we started by culturing five adherent, human epithelial cell lines: J82 (urinary bladder, transitional cell carcinoma), T24 (urinary bladder, transitional cell carcinoma), RT4 (urinary bladder, transitional cell papilloma), LNCaP (prostate carcinoma), and PC-3 (prostate, grade IV tumor, adenocarcinoma). These cell lines were targeted both because of their implication in accordance with GAMMA for LDOC1 association (refer to Table 1), and because of their availability as a result of Dr. Wren's collaboration with Dr. Robert Hurst of the Department of Urology at the University of Oklahoma Health Sciences

Userlist	Commonality	# Shared rels	Obs/Exp	Score	Lit Str
LDOC1	Pancreatic cancer	9	5.1	45	3.7
LDOC1	cell adhesion	10	3.96	38	0
LDOC1	epithelial-mesenchymal transition	5	7.77	38	0
LDOC1	telomerase activity	5	5.74	27	0
LDOC1	cell motility	5	3.83	18	0
LDOC1	cell differentiation	7	2.59	17	0
LDOC1	cell growth	8	2.07	16	0
LDOC1	cell death	8	2.06	16	0
LDOC1	mitogen-activated protein kinase	7	2.29	15	1.3
LDOC1	Prostate cancer	6	2.38	14	0.5
LDOC1	Angiogenesis	6	2.42	14	0
LDOC1	NF-kappaB	5	2.04	9	3.7

Table 1. Selections from GAMMA's Predicted Validated and Testable Associations. Validated associations highlighted the lighter shade.

Center (OUHSC). All cell lines were grown on plastic under sterile conditions in an incubator at 37˚C, 95% air and 5% carbon dioxide, using cellgro® Dulbecco's Modification of Eagle's Medium mixed with 10% Fetal Bovine Serum and 5.00 ml of cellgro® Penicillin/Streptomycin antibiotic, and harvested appropriately so as to maximize viability and ensure uncontaminated passage.

We isolated RNA from each of these cell lines using the Fujifilm QuickGene-Mini80 Nucleic Acid Isolation Device. We then verified RNA purity through the NanoDrop® ND-1000 Spectrophotometer, ensuring the 260 nm/280 nm absorbance spectra ratio for a 2.00 µl sample of RNA was at minimum 2.00, the accepted value for spectroscopic assertion of 0% protein contamination. Using this quality-tested RNA, we subsequently synthesized cDNA with the BIO-RAD iScript™ cDNA Synthesis Kit. We then plated this cDNA with LuminoCt® SYBR® Green qPCR ReadyMix™—and in some cases, iQ™ SYBR® Green Supermix—on a 96-well plate for single-step, Real-Time PCR (qPCR) evaluation. All reactions were performed in triplicates to ensure repeatability and precision of data collection, and each PCR plate had the cell lines plated with two sets of LDOC1 primers (LDOC1 set 1, LDOC1 set 2) and GAPDH primers for housekeeping. All plates had controls to verify that no reagent other than the cDNA from the cell lines contributed to the returned Ct values for gene amplification. The plates were sent to the Microgen Laboratory for Genomics and Bioinformatics at OUHSC for qPCR, and subsequently analyzed with Microsoft Excel. It should be noted that all qPCR data used in this study were normalized to GAPDH concentration for analysis.

We attempted to silence LDOC1 through two methods: ectopic transfection of Cy3-labeled siRNA oligos into harvested cells with siPORT™ NeoFX™ Transfection Reagent, and transfection through electroporation (hereafter called nucleofection) of Cy3-labeled siRNA oligos into harvested cells with Amaxa® Cell Line Nucleofector® Kit V. We verified LDOC1 silencing both qualitatively and quantitatively, by observing the transfected and nucleofected cells under a fluorescent microscope for "glowing" to indicate transfection, and by plating the cDNA synthesized from the transfected and nucleofected cells for qPCR to observe changes in Ct values.

For our final phenotypic analysis, we performed a wound-healing assay5 in which both silenced and untreated cells were plated in a 6-well plate, observed under a microscope for qualitatively determined silencing, and then "wounded." The wound consists of a cross, drawn with a 1000 µl pipet-tip in each well. The plate was observed and photographed at 0 hrs after wounding, 24 hrs after wounding, and 41 hours after wounding, for changes in behavior.

RESULTS

Different cell growth behaviors were noted for each cell line, which served as factors in our determination of which cell line to be ideal for the study. J82 and T24 cell lines proliferated extremely quickly, reaching optimal confluency (70-100%) within 1-2 days after plating; RT4 and PC-3 cell lines proliferated moderately, reaching optimal confluency within 5-7 days after plating; LNCaP cell lines grew extremely slowly,

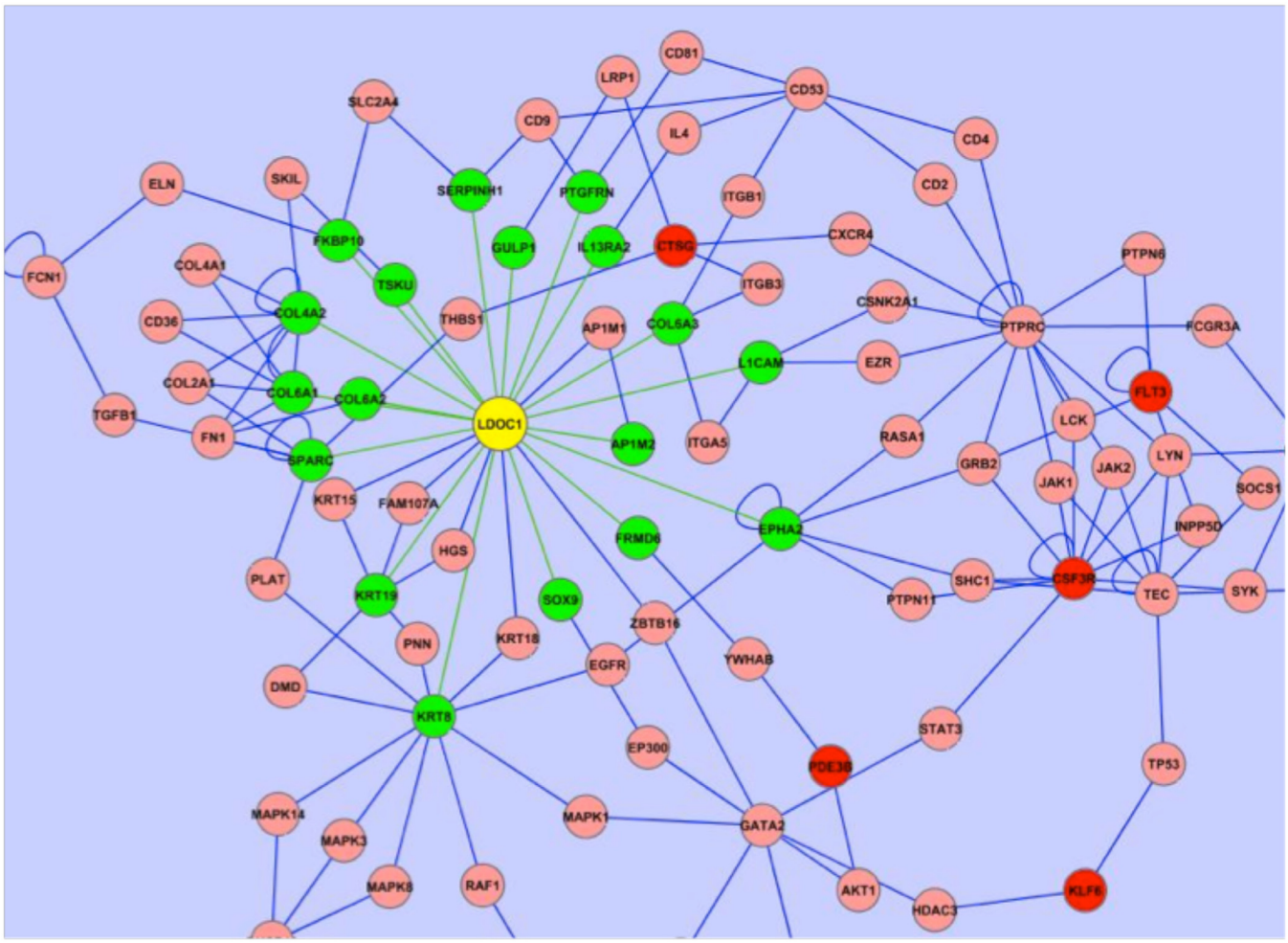


Figure 1. LDOC1 Hybrid Protein-Protein Interaction Network. LDOC1 is indicated in yellow. Green nodes indicate genes frequently co-expressed in parallel with LDOC1 (i.e., higher expression when LDOC1 expression is higher and lower expression when LDOC1 is low), red nodes indicate genes co-expressed in anti-parallel, and pink nodes indicate protein-protein interactions between these genes. Highly connected nodes suggest potential regulators and sub-group clusters suggest possible protein complex interactions or modifications.

reaching optimal confluency within 7-14 days after plating. RT4 cells grew in distinct clump formations; PC-3 and LNCaP cells grew in a similarly associative behavior, though not as distinctly clumped as RT4 cells; J82 and T24 cells demonstrated no such noticeable pattern of behavior. While J82, T24, RT4, and PC-3 cell lines were reasonably adherent and very easy to culture, LNCaP cell lines behaved erratically and had to be cultured with much sensitivity as they could very easily be removed from the plate. We were able to isolate quality RNA from all cell lines with ease, however.

We initially tested ectopic transfection as a viable means for LDOC1 silencing. Although via microscopy cells appeared to be effectively transfected, we observed through qPCR no noticeable change in amplification for LDOC1-silenced and LDOC1-expressed cells, indicative of ineffective silencing. We then turned to nucleofection as a method of silencing LDOC1, and found through microscopy that PC-3 cells were most effectively nucleofected. This was verified with qPCR results. Following, Figure 2 is a fluorescent microscope image of nucleofected PC-3 cells before qPCR, Figure 3 depicts the qPCR results from the nucleofected PC-3 cells, Figure 4 is a fluorescent microscope image of nucleofected PC-3 cells before the wound-healing assay, and Figure 5 shows the results of the wound-healing assay.

DISCUSSION

As stated before, we found nucleofection to be a better method than ectopic transfection for LDOC1 silencing. This was unexpected, as, though our chosen cell lines are difficult to transfect, nucleofection generally has lower cell viability. We suspect that the gentler, ectopic transfection method was ineffective because the siRNA transfection complexes were able to adhere to the cell membranes but unable to actually enter the cells; this cannot be very easily detected through microscopy (the cells will appear "glowing" regardless of where the siRNA complexes actually are in the cell) and is only quantifiable through qPCR. This is an issue which lends justification for the use of a more direct method like nucleofection, in which the cells, when subject to an electric field, open small pores to allow for the immediate injection of siRNA oligos. Regardless of the cell viability issues present with nucleofection, we found that PC-3 cells were most easily and most effectively nucleofected and LDOC1-silenced when compared to other cell lines, and this was confirmed both qualitatively and quantitatively.

The wound-healing assay yields promising results for targeted future investigation—we were able to see noticeable differences in the healing patterns and migration behavior of untreated and silenced cells. However, the assay still needs to be optimized. Contrary to expected patterns of behavior, cells from images 1, 2, and 3, in Figure 5 do not behave very similarly though all of them have LDOC1 expressed accordingly. We suspect that this is due to the effect of the

electric shock on the cells; although PC-3 cells were very successfully nucleofected and had optimal cell viability, the electric shock may induce delays in their ability to proliferate and migrate toward healing the wound. Also, the electric shock's impact on cell viability can be seen in a much more elemental sense: due to the electroporation, there may actually be fewer cells that are plated and wounded. We question further whether nucleofection affects other cell characteristics, like cell adhesion (recall that in image 1 in Figure 5, many cells had lifted themselves off of the plate). To optimize the assay, we propose titrating cell count and observing healing behavior and quantifying cell migration by measuring the distance the wound has closed at each time frame. We also suggest repeating the experiment to reach a more informed understanding of the behavior of LDOC1-silenced cells.

From a broader perspective, the study done on LDOC1 is valuable because it adds credibility to GAMMA's method for targeted investigation. This holds the potential for significant impact on scientific productivity—highly probable hypotheses can be identified and pursued. With the unbiased, mining capabilities of GAMMA, we can perform directed and informed studies, and broaden the realms of scientific development with knowledge we had previously left unrealized. We are therefore enabled, through pinpointed research, to more effectively uncover the biological roles of various, previously obscure, genes. Specifically relevant to LDOC1, researching further the phenomena discussed here (in terms of verifying protein expression, optimizing the wound-healing assay, repeating all experimental steps taken, etc.) can help to explain the role of LDOC1 to cancer in terms of cell migration and metastasis, and ultimately have impacts on medical treatments and diagnostics.

ACKNOWLEDGEMENTS

I would like to first express my gratitude to the Oklahoma Medical Research Foundation and its Sir Alexander Fleming Scholar Program for providing me the expansive opportunities I have received this summer to investigate these intriguing scientific challenges, and to Ms. Sharon Bell at the Richard K. & Ruth S. Lane Memorial Trust for sponsoring my scholarship. I cannot speak enough of the appreciation I have for my mentor, Dr. Jonathan Wren, and everyone in the Wren lab—Dr. Mikhail Dozmorov, Dr. Kristi Koelsch, Cory Giles,

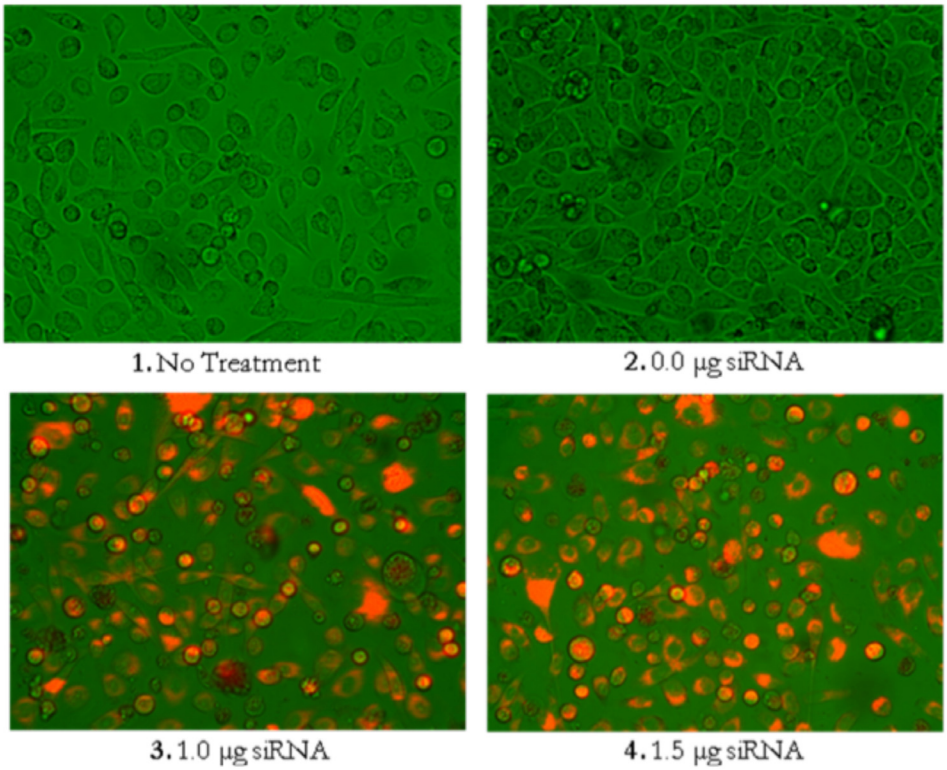
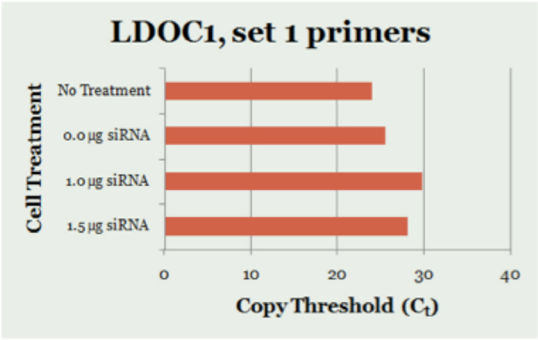
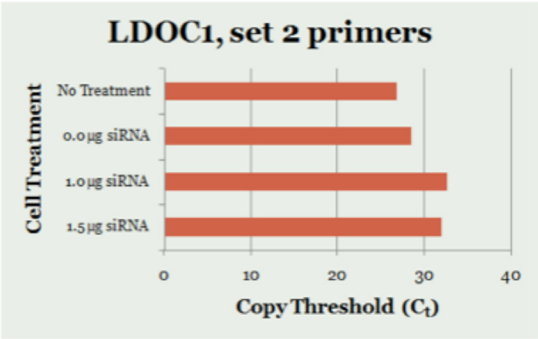


Figure 2. PC-3 cells, 24 Hours After Nucleofection and Before qPCR. 1. PC-3 cells were untreated and plated; 2. PC-3 cells were given AMAXA® Cell Line Nucleofector® Kit V Solution but no siRNA to control for any effects the solution would have on cell viability—note that cells actually appear more confluent; 3. PC-3 cells were nucleofected with 1.0 µg of siRNA; 4. PC-3 cells were nucleofected with 1.5 µg of siRNA. The bright orange seen in images 3 and 4 are the Cy3-labeled siRNA introduced into the cells, indicative of a successful nucleofection.



LDOC1, set 1 primers	
1.0 µg siRNA	98.31% LDOC1 silenced
1.5 µg siRNA	94.50% LDOC1 silenced



LDOC1, set 2 primers	
1.0 µg siRNA	98.33% LDOC1 silenced
1.5 µg siRNA	97.26% LDOC1 silenced

Figure 3. qPCR Results of Nucleofected PC-3 Cells. Note that for both LDOC1, set 1 primers and LDOC1, set 2 primers, there is a distinct difference in Ct values for cells that had been nucleofected with siRNA and cells that had been untreated, and that the use of 1.0 µg siRNA was optimal for silencing in both cases. Silencing percentages calculated through ddCt method.

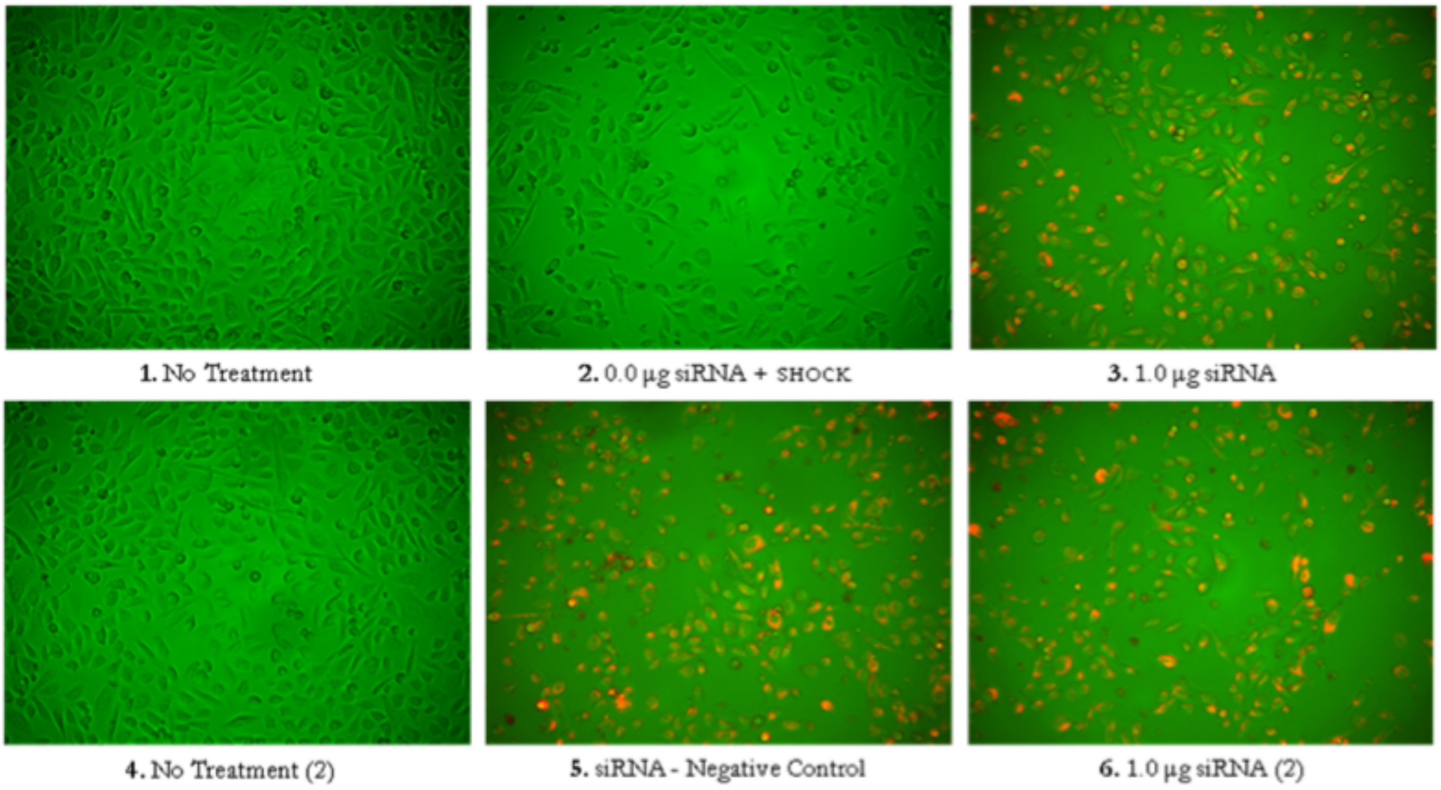


Figure 4. PC-3 cells, 22 Hours After Nucleofection and Before Wound-Healing Assay. 1, 4. PC-3 cells were untreated and plated; 2. PC-3 cells were given AMAXA® Cell Line Nucleofector® Kit V Solution and were electroporated, but given no siRNA, serving to control for any effects the electric shock has on cell viability—note that in image 2, the cells are significantly less confluent than in images 1 and 4; 3, 6. PC-3 cells were nucleofected with 1.0 µg of siRNA; 5. PC-3 cells were nucleofected with 1.0 µg of scrambled siRNA, serving as a negative control for unintended "silencing." Once again, note the bright orange in images 3, 5, and 6 from the Cy3-labeled siRNA, qualitatively indicative of a successful nucleofection.

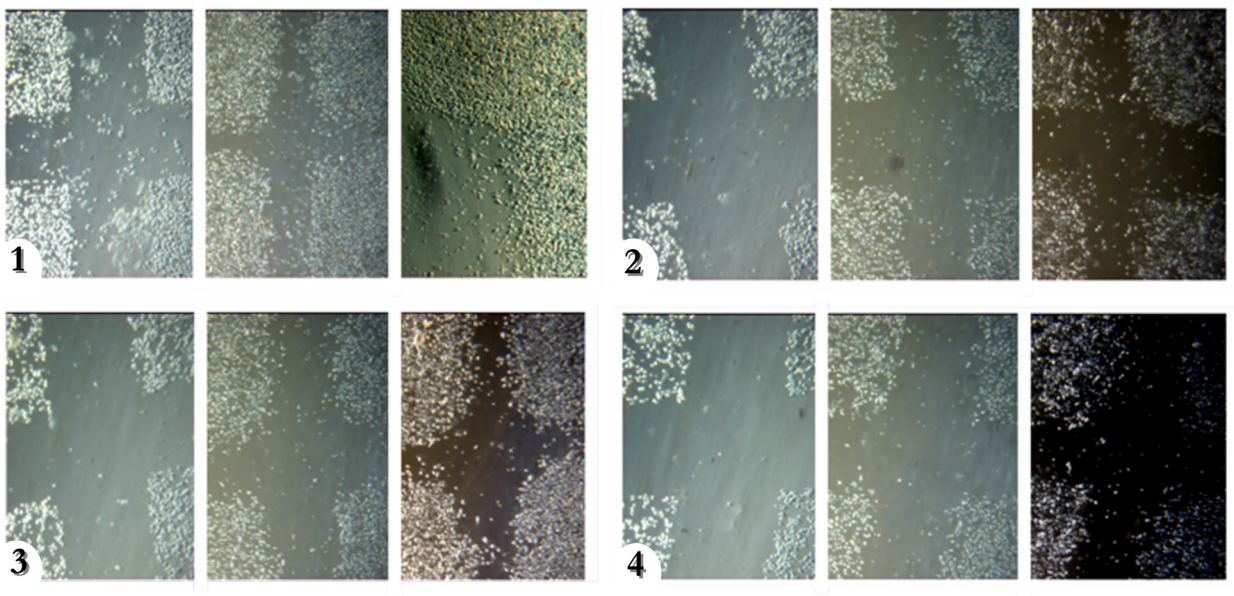


Figure 5. Summary of the Wound-Healing Assay. Wound-healing assay of nucleofected PC-3 plate, each well a set of 3 images, progressing horizontally at 0, 24, and 41 hours. First row, left to right: 1. No treatment , 2. 0.0 µg siRNA + shock; second row, left to right: 3. 1.0 µg siRNA negative control , 4. 1.0 µg siRNA. Note that in each set of 3 images, the cells have reacted distinctly differently to the wound. In 1, the cells have completely closed the wound, but by 41 hours, more than half of them are freely floating, not adherent to the plate. In 2, we notice the cells gradually attempting to close the wound, though not as dramatically as 1. In 3, we notice a much more distinct attempt to close the wound, and it is evident that the cross is shrinking because cells are rapidly proliferating. In 4,we notice a much more gradual attempt to close the wound, with the cross at 41 hours only slightly smaller than the cross at 0 hours.

and Travis Chapman—for providing me guidance, support, and insight in the procedure of my experimentation wherever possible. A huge thank you to Dr. Robert Hurst for allowing me the facilities of his laboratory, and to Ms. Jean Coffman, Dr. Danuta Sosnowska, and Dr. Paul Hauser for providing me ready assistance with every technique I needed to learn or improve upon. Lastly (though in no way least), I would like to thank Ms. Courtney Stevens, Ms. Ann Sullivan, Oklahoma City University, and everyone who helped to make this program possible, both personally and professionally.

REFERENCES

1. Nagasaki K, Manabe T, Hanzawa H, Maass N, Tsukada T, Yamaguchi K. Identification of a novel gene, LDOC1, down-regulated in cancer cell lines. Cancer Lett. 1999 Jun 1;140(1-2):227-34.
2. Inoue M, Takahashi K, Niide O, Shibata M, Fukuzawa M, Ra C. LDOC1, a novel MZF-1-interacting protein, induces apoptosis. FEBS Lett. 2005 Jan 31;579(3):604-8.
3. Nagasaki K, Schem C, von Kaisenberg C, Biallek M, Rösel F, Jonat W, Maass N. Leucine-zipper protein, LDOC1, inhibits NF-kappaB activation and sensitizes pancreatic cancer cells to apoptosis. Int J Cancer. 2003 Jul 1;105(4):454-8.
4. Wren JD. A global meta-analysis of microarray expression data to predict unknown gene functions and estimate the literature-data divide. Bioinformatics. 2009 Jul 1;25(13):1694-701. Epub 2009 May 15.
5. Wallert and Provost Lab. Wound Assay Protocol. http://www.mnstate.edu/provost/woundAssayProtocol.pdf. Aug 6, 2010.