ROLE OF THE SCAFFOLDING PROTEIN p62Dok IN INVASIVENESS OF Src-TRANSFORMED CELLS

A Senior Scholars Thesis

by

RYAN WILLIAM TURKINGTON

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2011

Major: Biomedical Science

ROLE OF THE SCAFFOLDING PROTEIN p62Dok IN INVASIVENESS OF Src-TRANSFORMED CELLS

A Senior Scholars Thesis

by

RYAN WILLIAM TURKINGTON

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by:

Research Advisor: Gonzalo M. Rivera
Director for Honors and Undergraduate Research: Sumana Datta

April 2011

Major: Biomedical Science

ABSTRACT

Role of the Scaffolding Protein p62Dok in Invasiveness of Src-Transformed Cells.
(April 2011)

Ryan William Turkington
College of Veterinary Medicine and Biomedical Science
Texas A&M University

Research Advisor: Dr. Gonzalo M. Rivera
Department of Veterinary Pathobiology

Tumor cells use actin-based structures capable of degrading the extracellular matrix during the process of invasion and metastasis. Recent studies demonstrate that the adaptor protein Nck-2, an important link between tyrosine phosphorylation and cytoskeletal remodeling, is required for invasion in cancer cells. Using biochemical approaches, we have previously uncovered that the scaffolding protein p62Dok interacts with Nck in transformed (tumor-like) but not normal cells. However, we know very little about this interaction and the role of p62Dok in mechanisms of invasion of cancer cells. We seek to determine if this interaction is important in living cells. We used a combination of molecular genetics, proteomics, and high-resolution optical microscopy to determine the subcellular distribution of p62Dok and to determine if p62Dok is required for tumor cell invasion. We have successfully induced invasive structures in fibroblasts and have made a fluorescent Dok1 fusion protein. Preliminary data suggest

that Dok1 localizes to these invasive structures. Studies are ongoing to prove conclusively that Dok1 localizes to invasive structures.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I
INTRODUCTION

Podosomes and invadopodia are actin-based structures that are involved in metastatic invasion of cancer cells (Stylli et al., 2008). These invasive structures can be induced by introducing a constitutively active form of the Src protein. Src is involved in cytoskeletal remodeling. It is an oncogene which commonly has elevated activity in cancer cells (Stylli et al., 2009). Src-transformed fibroblasts have an aberrant morphology and the podosomes can be visualized by staining filamentous actin with fluorescently labeled phalloidin. The stained actin in Src-transformed cells forms circular 'rosettes' which are indicative of podosome formation (Winograd et al., 2011). The adaptor protein Nck-2 has been localized via fluorescent confocal microscopy to podosomes which suggest that Nck-2 has a role in the formation of podosomes (Stylli et al., 2009). It has recently been determined via far western blot that Nck-2 and Downstream of Tyrosine Kinase protein 1 (Dok-1, p62 Dok) interact with in Src-transformed fibroblasts, but not in normal fibroblasts. The purpose of this research project is to determine if Dok-1 is required for podosome and invadopodia formation. To transform normal fibroblasts to Src-transformed fibroblasts, a retrovirus was made with a constitutively active mutant Src construct. A combination of fluorescent microscopy techniques including confocal immunofluorescence and TIRF imaging with a Dok-1/YFP fusion protein were used to determine the subcellular localization of Dok-1 and to determine if Dok-1 colocalizes

---

This thesis follows the style of *Journal of Cell Biology*.

with podosomes. Localization of Dok-1 to podosomes would suggest that it plays a role in podosome formation in Src-transformed cells.

CHAPTER II

METHODS

Creation of Dok1-GFP fusion protein

Primers for a polymerase chain reaction (PCR) were designed for use on an existing Dok1 template. After the PCR was run, the amplified DNA was digested with the restriction enzymes Not1 and BglII. The parental vector with a GFP (MSCV) insert was also digested with the same restriction enzymes. Both DNA constructs were purified using agarose gel electrophoresis. The parental vector and PCR product were then ligated with T4 ligase. Competent bacteria (E. coli K12) were then transformed using the product of the ligation and were selected with ampicillin. Single colonies were picked and grown in LB broth with ampicillin overnight. These bacteria were centrifuged and processed to separate the DNA from other cellular components. The plasmid DNA was then separated from the chromosomal DNA by ultracentrifugation with cesium chloride. Next, a retrovirus was prepared by transfecting 293T cells with the Dok1-GFP construct and the virus components gag.pol and env. The media containing the virus particles was collected at regular intervals and frozen at -80° C. Two types of NIH 3T3 cells were cultured for infection with the virus. Normal 3T3 cells were cultured as a control and cells with a mutated Src protein were cultured. The mutant Src protein induces podosome formation in 3T3 cells. The infected cells were then selected using puromycin. Expression of the fusion protein was confirmed with fluorescent microscopy and western blot.

Imaging

The cells expressing the Dok1-GFP were seeded on a glass bottom Matek plate coated with fibronectin for total internal reflection fluorescence (TIRF) microscopy, a type of microscopy that visualizes fluorescence at the cell membrane. The TIRF was done in the imaging laboratory at the vet school. Immunofluorescence was also done to visualize colocalization with actin and Dok1. Cells were first seeded on coverslips coated with poly-D-lysine. Cells were then fixed with paraformaldehyde and permeabilized with Triton-X100. The cells were blocked with 1% BSA and incubated with an α-Dok1 antibody. Then they were incubated with an α-IgG secondary antibody mixed with DAPI to visualize the nucleus and Texas red phalloidin to visualize filamentous actin. The coverslips were mounted with fluorescein and taken to the imaging laboratory for the fluorescent microscopy. LSM software was used to merge the images and determine colocalization.

FACS Sorting

Cells were infected with the pLenti lentivirus and the cultures were expanded. The confluent cultures were trypsinized, centrifuged, and resuspended in PBS. The suspensions were taken to the flow cytometry facility at the Texas A&M Health Science Center. Cells were analyzed with a 488 nm laser. Cells that had a fluorescence peak were collected and expanded in culture.

CHAPTER III

RESULTS

Cloning

We initially attempted to make a fusion protein of Dok-1 using the MSCV retroviral system. We were unable to use the MSCV vector to infect 3T3 fibroblasts so we cloned Dok-1 into the pLenti lentiviral system. We were able to produce clones in the pLenti vector. Agarose gel electrophoresis confirmed that Dok1 was cloned into the vector (see Fig. 1).

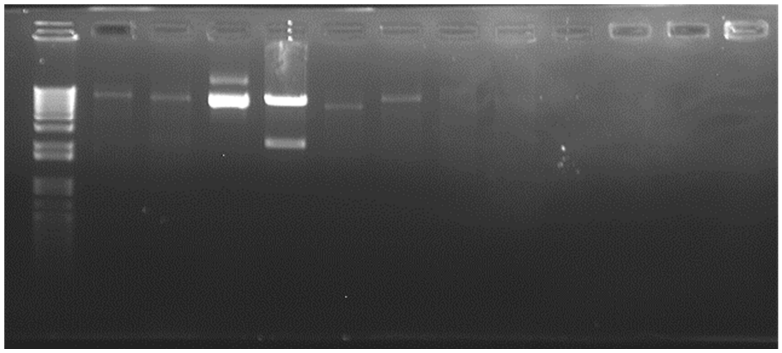

Figure 1 Agarose Gel Electrophoresis of the pLenti-Dok-1 Clone. Lane 1 is the ladder. Lane three is the clone. The expected size of the clone is 9801 bases. The lighter band is due to supercoiling of the uncut plasmid. Lane 4 is the clone digested with the restriction enzymes NotI and SmaI. The linear vector should have a size of 7606 bases, while the insert should have a size of 2195 bases. The other lanes contain negative clones.

To test the expression of the Dok-1 fusion protein, 293T cells were chemically transfected to express the protein. The cells were then harvested and the lysates were analyzed using a western blot. To check the protein, we used an antibody that recognizes the EYFP tag on the fusion protein, and an antibody that is specific to human Dok-1. The lysates were run on an SDS-PAGE gel and the protein was transferred to a nitrocellulose membrane. The membrane was cut so that we could analyze the sample with both antibodies. Both samples were blocked in 0.5% non-fat dry milk and were then incubated with the appropriate antibodies and secondary antibodies. An antibody against β-actin was included on one of the membranes as a loading control to ensure that equal amounts of protein were added to each lane. A chemiluminescent reagent was then added to the membrane and images were taken using the ImageQuant system. The western blot using an antibody specific to Dok1 and the fluorescent tag confirmed that our fusion protein was being expressed as we intended (see Fig. 2).

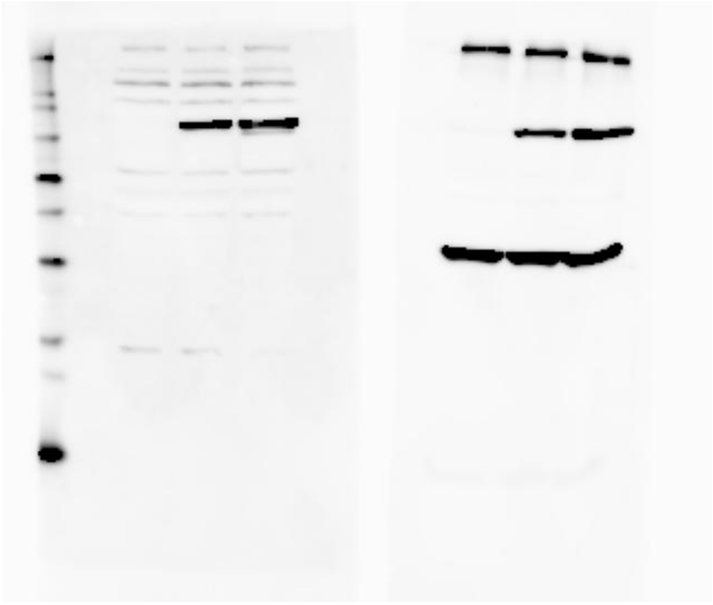

Figure 2 p62Dok-EYFP fusion protein western blot. 293T lysates were run using SDS-PAGE electrophoresis. The exposure time was 30 seconds. After the ladder, the first lane contained 293T lysates transfected with no plasmid. Lane two contained lysates transfected with 2 µg of plasmid and the lysates in lane 3 were transfected with 5 µg of plasmid. The membrane on the left was incubated with α-Dok-1 polyclonal antibodies and the two prominent bands indicate that overexpression of Dok-1 occurred in the cells that were transfected with the Dok-1 fusion protein, but not in the control cells. The membrane on the right was incubated with the β-actin loading control and a α-GFP antibody. The two prominent bands indicate that the fluorescent protein was expressed with the Dok-1 protein confirming the positive clone.

Podosome induction with c-Src Y530F

To induce podosomes in the 3T3 fibroblasts, virus was prepared to induce the production of a constitutively active form of the Src protein. The tyrosine to phenylalanine mutation in the 530th residue renders the protein constitutively active and a retroviral vector

containing the mutant protein and puromycin resistance was already available. Viruses were prepared by transfecting 293T cells with the MSCV(puro)-Y530F c-Src plasmid, and the DNA constructs needed to make a retrovirus. 3T3 cells were then infected with the c-Src retrovirus and selected for using puromycin to kill the uninfected cells. After the pharmacological selection, the cells infected with the virus remained healthy while naïve cells were completely killed with the drug selection. To confirm podosome formation, the cells were seeded on a coverslip for immunofluorescence. Cells were fixed with 4% paraformaldehyde and incubated with antibodies against cortactin, a known podosome marker. The cells were also incubated with Texas-Red phalloidin, a stain for filamentous actin. The cells were also stained with DAPI to visualize the nucleus. The coverslips were then mounted and visualized with a 510 META NLO laser scanning microscope. The images were overlaid using LSM software for confocal imaging showing the colocaization of actin and cortactin in the podosomes (see Fig. 3).

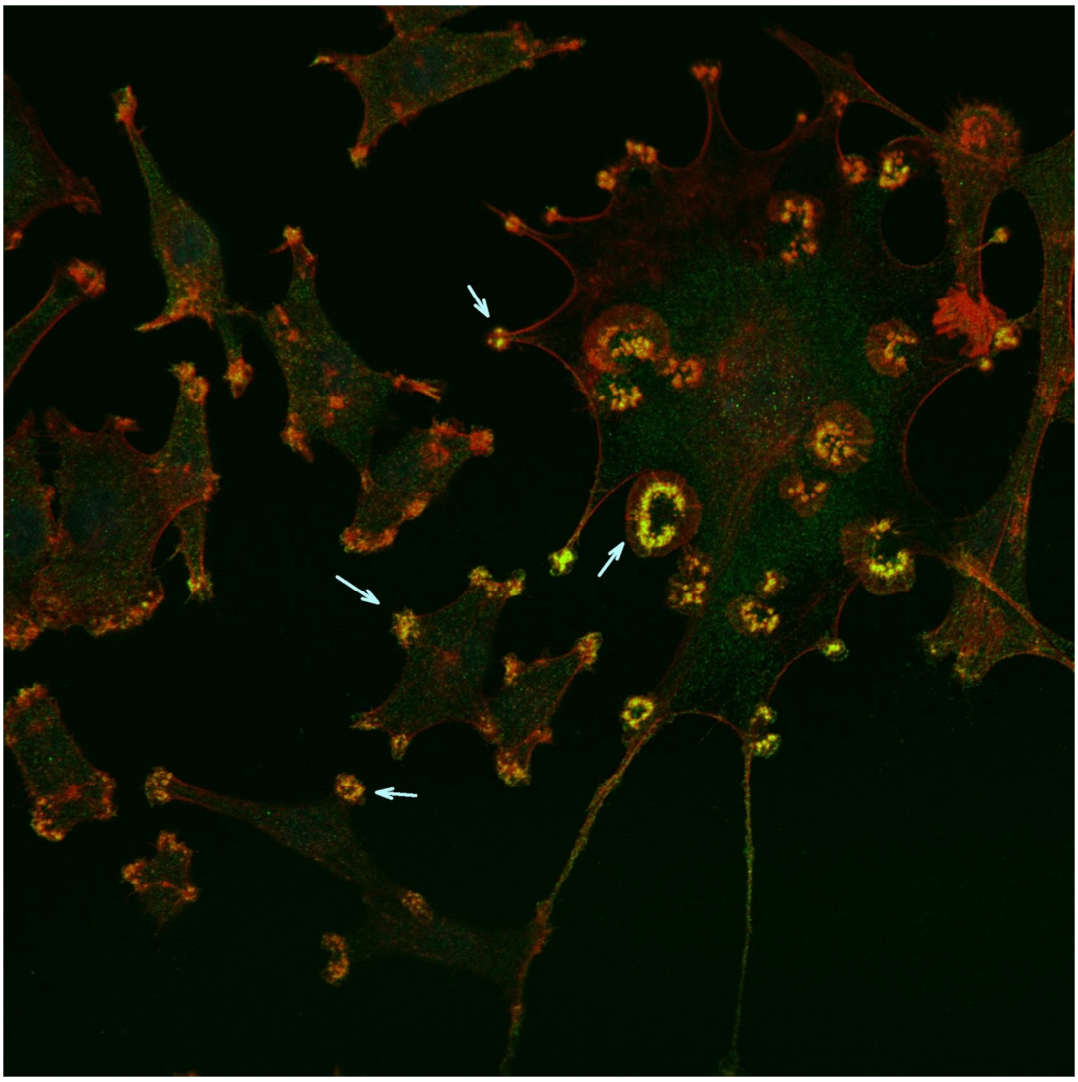

Figure 3 Confocal imaging of podosome production. c-Src 3T3 cells were stained with a cortactin antibody (green), Tx-Red phalloidin (red), and DAPI (blue). Yellow indicates colocalization between cortactin and actin, marking a podosome. Arrows are pointing to a selection of the podosomes in the image.

TIRF imaging

So far, TIRF imaging has had mixed results. Cells infected with the mCherry-Actin fusion protein do show strong localization of actin to podosomes. Due to low infection

efficiency of the pLenti virus system, we have been unable to take images of cells expressing both the mCherry –Actin and Dok-1-YFP fusion proteins. We are working to increase the infection efficiency of the virus and by sorting the infected cells using flow cytometry. We have had success with the cell sorting. The efficiency of the infection was 5%, but we were able to sort the cells to 95% purity.

CHAPTER IV

SUMMARY

Podosome induction

The cell lines that were infected with the c-Src Y530F virus consistently formed podosomes and exhibited other hallmarks of tumor cells, such as loss of contact inhibition. Immunofluorescence showed colocalization of actin with the podosome marker cortactin confirming the formation of podosomes in the c-Src 3T3 fibroblast cells. The formation of podosomes in these cells allows us to study the physiology of invasive cancer cells.

Fusion protein creation and TIRF imaging

We encountered a problem with the creation of a Dok-1-GFP fusion protein with the MSCV retroviral system. We changed virus systems to use the pLenti system. We successfully made the clone in the pLenti system and confirmed the expression of the protein with western blot. We optimized the use of the pLenti virus system and then infected cells with the new virus. Attempts to visualize colocalization between actin and Dok-1 using TIRF were unsuccessful because of the low infection efficiency of the virus. The success of the cell sorting will allow us to view any colocalization between the two proteins with the TIRF system.

Future research

We first noted the interaction of Dok-1 and Nck-2 with a far western blot using the SH2 domain of Nck. The SH2 probe localized to Dok-1 on the nitrocellulose membrane (size

62 kDa). We will confirm this interaction by immunoprecipitation. We will lyse normal 3T3 cells and 3T3's expressing the mutant Src protein. Then we will precipitate the Nck-2 protein with an αNck-2 antibody. Then a western blot will be run with the precipitant and the membrane will be probed with an αDok-1 antibody to determine if Dok-1 was pulled down with Nck-2.

Podosome formation is important to the release of matrix metalloproteinases that degrade the extracellular matrix to allow the cells to metastasize, or invade other tissues. Future studies of the role of Dok-1 in the formation of podosomes, and by extension the molecular mechanism of metastatic invasion, will involve knockdown of Dok-1. We will use small interfering RNA (siRNA) to prevent translation of Dok-1 mRNA in c-Src 3T3 cells. We will also introduce a point mutation to the Dok-1 protein (Y362F) that will prevent phosphorylation of the tyrosine residue that will render the protein inactive. The mutant protein will then be overexpressed in 3T3 fibroblasts to make the cells dominant negative for Dok-1. The dominant negative Dok-1 should have similar actin dynamics to the Dok-1 siRNA KD. These two approaches to stop Dok-1 activity in the cultures will confirm the physiological effects of Dok-1 on podosome formation. If the cells have a significant reduction in their ability to form podosomes, then we will show that Dok-1 is a key player in the formation of podosomes. We will also study the degradation of a fluorescently labeled matrix quantitatively to determine if the loss of Dok-1 prevents the degradation of the extracellular matrix by matrix metalloproteinases.

REFERENCES

Stylli, S.S., A.H. Kaye, and P. Lock. 2008. Invadopodia: at the cutting edge of tumour invasion. *J. Clinical Neuroscience*. 15:725-737.

Winograd, S.E., M.C. Brunner, N. Mirlas, B. Geiger. 2011. Analysis of the signaling pathways regulating Src-dependent remodeling of the actin cytoskeleton. *Eur. J. Cell Biol*. 90:143-56.

Supplemental sources consulted

Hosooka, T., T. Noguchi, H. Nagai, T. Horikawa, T. Matozaki, M. Ichihashi, and M. Kasuga. 2001. Inhibition of the motility and growth of B16F10 mouse melanoma cells by dominant negative mutants of Dok-1. *Molecular and Cellular Biol.* 21:5437-5446.

Niu, Y., F. Roy, F. Saltel, C. Andrieu-Soler, W. Dong, A.L. Chantegrel, R. Accardi, A.Thépot, N. Foiselle, M. Tommasino, P. Jurdic, and B.S. Sylla. 2006. A nuclear export signal and phosphorylation regulate Dok1 subcellular localization and functions. *Molecular and Cellular Biol*. 26:4288-4301.

Oser, M., H. Yamaguchi, C.C. Mader, J.J. Bravo-Cordero, M. Arias, X. Chen, V. DesMarais, J. van Rheenen, A.J. Koleske, and J. Condeelis. 2009. Cortactin regulates cofilin and N-WASp activities to control the stages of invadopodium assembly. *J. Cell Biol*. 186:571-587.

Owen, K.A., F.J. Pixley, K.S. Thomas, M. Vicente-Manzanares, B.J. Ray, A.F. Horwitz, J.T. Parsons, H.E. Beggs, E.R. Stanley, and A.H. Bouton. 2007. Regulation of lamellipodial persistence, adhesion turnover, and motility in macrophages by focal adhesion kinase. *J.Cell Biol*. 179:1275-1287.

Rottiers, P., F. Saltel, T. Daubon, B. Chaigne-Delalande, V. Tridon, C. Billottet, E, Reuzeau, and E. Génot. 2009. TGFβ-induced endothelial podosomes mediate basement membrane collagen degradation in arterial vessels. *J. Cell Sci*. 122:23.

Smith, A., J. Wang, C.M. Cheng, J. Zhou, C.S. Weickert, and C.A. Bondy. 2004. High-level expression of Dok-1 in neurons of the primate prefrontal cortex and hippocampus. *J. Neuroscience Research*. 75:218-224.

CONTACT INFORMATION

Name:	Ryan William Turkington
Professional Address:	c/o Dr. Gonzalo Rivera Department of Veterinary Pathobiology MS 4467 Texas A&M University College Station, TX 77843
Email Address:	ryanwturkington@gmail.com
Education:	B.S. Biomedical Science, Texas A&M University, August 2010 Undergraduate Research Scholar