SI Appendix – A Role for PAK4 in A549 Tumor Cell Biology.

Results for Role of PAK4 in A549 Tumor Cell Biology.

The role of PAK4 in A549 NSCLC tumor cells was studied using siRNA and antisense oligomer (ASO) knockdown methodologies. A549 cells were found to have a significant level of PAK4 mRNA and protein in the untreated cells which could be knocked down by either ASO or siRNA methodologies.

Antisense oligomer PAK4 knockdown was used to validate that A549 cells are PAK4-dependent. Antisense oligomers for PAK4 caused a large decrease in PAK4 mRNA and protein levels relative to the untransfected control A549 cells (UTC) (Figure A). The non-specific ASO (N20) did not have a measurable effect relative to the UTC.

PAK4 ASO treatment of A549 cells was evaluated to assess PAK4 dependent functional effects. Pak4-ASO treatment of A549 was performed in either growth media or serum free conditions and plated in soft agar @ 48h. Colonies were colonies counted at day 14. PAK4 ASO caused a significant (75 – 95%) decrease in the total number of colonies (Figure B).

Next, PAK4 ASO was used to assess poly-caspase activity through FAM-VAD binding and measured via cytometry (Figure C). PAK4 ASO cause a large increase in the amount of activated caspase compared to the control ASO (N20) at both the 24 and 48 hour time points. After 24h of ASO treatment, the relative fraction of activated caspase was 11.8 for the PAK4 ASO treatment while the control ASO (N20) was 4.8. After 48 of ASO treatment, the PAK4 ASO had a 25.7 relative fraction of cells with activated caspase while the control ASO had only 4.43.

PAK4 siRNA knockdown of PAK4 was evaluated (Figure D & E). Robust PAK4 knockdown at the protein level was achieved 72h after PAK4 siRNA treatment but not mock treated or control siRNA. Complete knock down of endogenous PAK4 expression by was seen in A549 cells transfected with a concentration range of 67 to 100nM of PAK4 RNAi #732 [sense 5’ GGGACUACCAGCACGAGAA 3’] (results were confirmed with a second independent PAK4 RNAi #733 [5’ GGUGAACAUGUAUGAGUGU 3’]). A549 cells were treated with PAK4

siRNA (100 nM) or siRNA controls (mock treated, risk-free siRNA, non-targeting siRNA) for 72h. PAK4 siRNA affected a significant onset of apoptosis with 45% of A549 cells (n= 9 wells) had activated caspase-3 (Figure D). There was no significant increase in caspase-3 activation in the control samples (n=9 each): 0.20% (mock treated), 0.29% (control siRNA at 50nM) and 0.23% (control RNAi at 100nM). For control siRNAs, we either used a non-targeting RNAi or 'risk free' RNAi; in either case we didn't see a significant difference from mock controls with either control siRNAs (both control siRNAs gave <0.29% caspase 3 activated A549 cells at 72 hrs). When pyrrolopyrazole inhibitors related to PF-3758309 were tested (earlier generation compounds), significant caspase-3 activation was observed within 33h: 30% of A549 cells (1 μM PF-808119, PAK4 K_i = 29 nM, phosphoGEF-H1 IC_{50} = 29 nM) and 56% of A549 cells (1 μM PF-834067, PAK4 K_i = 15 nM, phosphoGEF-H1 IC_{50} = 16 nM). PAK4 siRNA was used to study the effect of PAK4 on A549 cell morphology. PAK4 siRNA treated and control siRNA treated cells were phalloidin-stained after 72h (Figure E). A significant change of the actin cytoskeleton was observed with PAK4 siRNA treatment compared to both untreated control cells and control siRNA treatment.

Figures for the Role of PAK4 in A549 Tumor Cell Biology

Figure A.

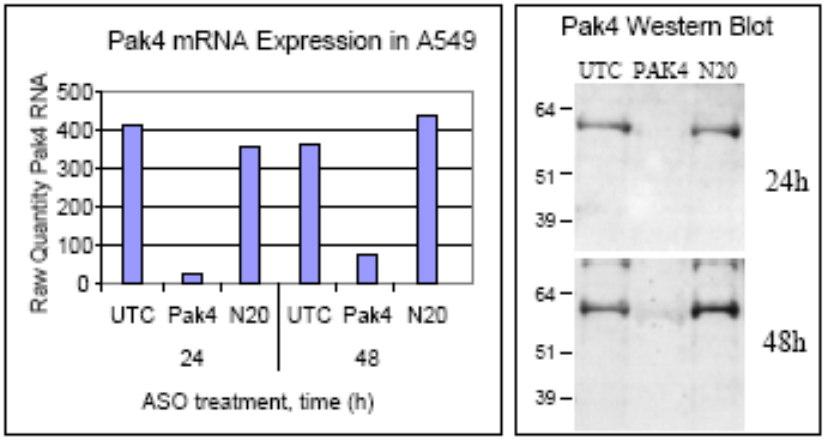

Figure B.

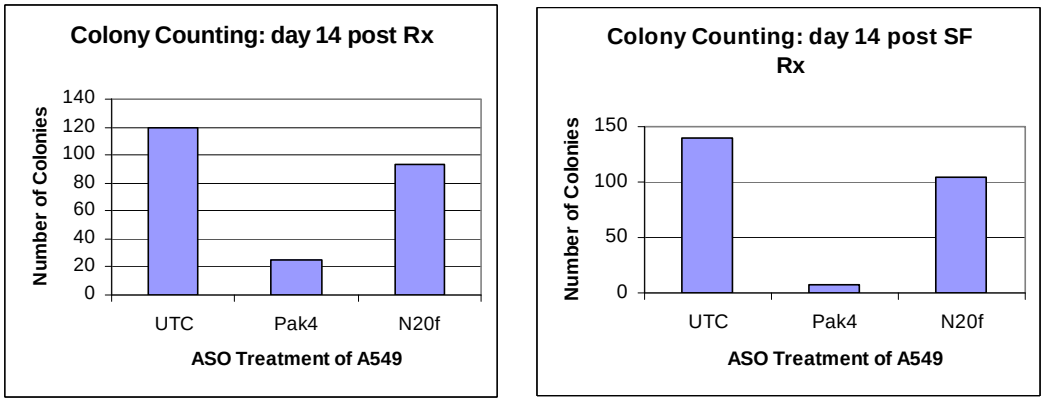

Figure A & B. Antisense mediated knockdown of PAK4 in A549 cells inhibits soft agar colony formation. A549 cells transfected with PAK4 or control (N20) antisense oligonucleotides were analyzed for PAK4 mRNA levels using rtPCR and PAK4 protein levels via western blotting (A). Antisense treated cells in soft agar colony formation assays using serum containing (right panel) and serum free (serum free) conditions (B). UTC = untreated cells. Results are representative of two separate experiments in which PAK4 depleted cells showed greater than 80% reduction in colony formation.

Figure C.

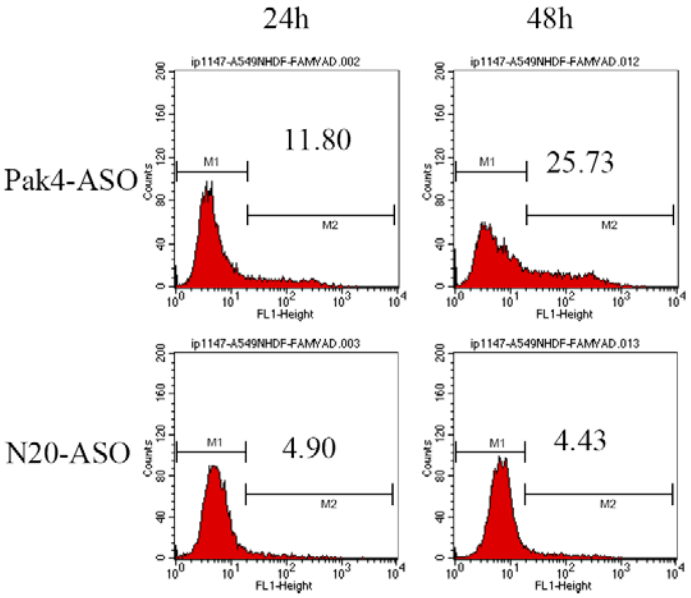

Figure C. Antisense mediated knockdown of Pak4 in A549 cells promotes Caspase activation. Pan-Caspase activity in A549 cells transfected with control (N29-ASO) and Pak4 specific antisense oligonucleotides. Flow cytometric analysis of cells stained with the pan-Caspase probe FAM-VAD-FMK 24 and 48 hours post antisense transfection. The percentage of cells staining positive for Caspase activity are noted in each histogram. Results are representative of three independent experiments.

Figure D

72 Hours after Transfection	% Apoptotic cells	(Caspase 3 activation)		
	mock	10nM	50nM	100nM
Control RNAi (N=9)	n/a	ND	0.29 +/- 0.19	0.23 +/- 0.15
PAK4 RNAi #732 (N=9)	n/a	0.21 +/- 0.10	1.94 +/- 1.15	45 +/- 15.69
Mock(N=9)	0.20 +/- 0.17			

Figure E

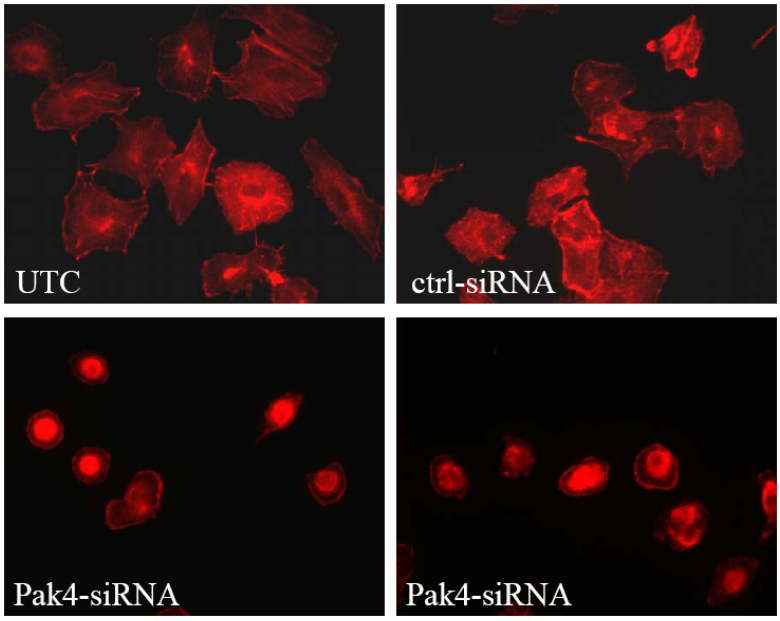

Figure D & E. PAK4 knockdown in A549 cells affects cytoskeletal regulation. (D) PAK4 siRNA caused A549 cell apoptosis relative to control siRNA. (E) Phalloidin staining of untreated (UTC) A549, and cells transfected with control siRNA or PAK4 specific siRNA. Cells were replated 24 hr following transfection and allowed to adhere for 24 hours before phalloidin staining and image acquisition at 32X.

Methods for Role of PAK4 in A549 Tumor Cell Biology.

Antisense Oligonucleotide Treatments of A549 Cells. A549 cells were grown to 60-70% confluence in T-75 flasks. The cells were then washed twice with Dulbecco's modified Eagle's medium and then 5 ml of Dulbecco's modified Eagle's medium containing 20 µg/ml N-[1-(2, 3-dioleyloxy)propyl]-n, n,n-trimethylammonium chloride/dioleoyl phosphatidylethanolamine (DOTMA/DOPE) (Lipofectin®)(Life Technologies, Inc.) solution was added to the flasks. Oligonucleotides were added to the required concentration from a 10 µM stock solution and the flask swirled to mix the solutions. The cells were then incubated at 37° C for 4 h and then the DOTMA/DOPE/oligonucleotide solution was aspirated off and replaced with medium for the indicated time.

siRNA Treatment of A549 Cells. The siRNA was synthesized by Ambion (Austin, TX). The primary siRNA (732) was as follows: 5'-GGGACUACCAGCACGAGAAtt (sense) and 5'-UUCUCGUGCUGGUAGUCCCtc (antisense). The confirmatory siRNA (733) was as follows: 5'-GGUGAACAUGUAUGAGUGUtt (sense) and 5'-ACACUCAUACAUGUUCACCtc (antisense). A549 cells were transfected with siRNA with the DharmaFECT transfection reagent per the manufacture's protocol (Thermo Scientific, Lafayette, CO).

Soft agar colony formation. A suspension of 1% agar (w/v) was heated in a microwave until molten and a bottom layer (0.5% agar) was prepared by coating a 60 mm dish with 1.5 ml of a 1:1 mixture of 2x DMEM (with or without serum), and 1% agarose. A top layer of agar was prepared (0.35% agar) by adding a 1:1 mixture of 2x DMEM (with or without serum) and 0.7% agarose containing 25,000 cells. Plates were then incubated for 14 days and colonies were stained using methylene blue and counted using the automated colony counting application on a Bio-Rad Fluor-S MultiImager.

Caspase activation: ASO transfected cells were harvested at the indicated time points following transfection and stained for pan-Caspase activity using the CaspaTag Pan-Caspase kit (Millipore) according to manufacture instructions. Caspase positive cells were detected via cytometry on a FacsCaliber flow cytometer (BD). A complementary method was used to assess caspase activation. The Cellomics apoptosis HitKit (Pittsburgh, PA) was used to quantify the amount of

caspase activation per the manufacture's protocol. The primary antibody (4 μg/mL) was purchased (BioVision, Mountain View, CA, catalog #3015-100) and used with a 1:50 dilution. The secondary antibody (goat anti-rabbit IgG) was purchased from Molecular Probes (Invitrogen, Carlsbad, CA, catalog number A11034) and used at a 1:1500 dilution. Imaging was performed on a Cellomics ArrayScan instrument using the Target Activation application.

Phalloidin staining

A549 cells were transfected with PAK4 and control siRNA as described and at 24 hr post transfection cells were harvested via trypsinization and replated in 4-well side chambers (Nunc) in complete medium conditions. Following 24 hr incubation at 37º C cells were fixed with 3.7% paraformaldehyde and stained with Alexa Fluor 568 phalloidin (Invitrogen) according to manufacturer's instructions.