Online Data Supplement

Nuclear β-catenin is increased in SSc Pulmonary Fibrosis and Promotes Lung Fibroblast Migration and Proliferation

Anna P. Lam[1#], Annette S. Flozak[1], Susan Russell[1], Jun Wei[2], Manu Jain[1], Gökhan M. Mutlu[1], G. R. Scott Budinger[1], Carol A. Feghali-Bostwick[3], John Varga[2]
and Cara J. Gottardi[1#]

Methods

Cell Culture and Antibodies-

Normal human lung fibroblasts (Clontechs NHLFs; Lot number 4F0758) were obtained from an 11 year old female by Lonza Walkersville Inc. (Walkersville, MD). Human lung fibroblasts (NL-57) from normal lung tissues obtained from organ donors under a protocol approved by the University of Pittsburgh Institutional Review Board were cultured in Dulbecco's modified Eagle's medium (Sigma, Dorset, United Kingdom) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and an antimycotic agent as described previously(1). Adult lung fibroblasts were maintained in Fibroblast Growth Medium (Lonza) with or without serum, as indicated. Human WI-38 diploid fibroblastic cells were originally derived from normal embryonic (3 months gestation) lung tissue and obtained from ATCC (CL-75; Manassas, VA). WI-38 cells were propagated in MEM-10%FBS until confluent, changed to differentiation medium (DM-2-L1, Zenbio, Research Triangle Park, NC) for three days, then changed to maintenance medium (AM-1-L1, Zenbio) for three days, and finally infected with adenovirus in MEM-10%FBS for 24 hours (RNA) or 48 hours (Protein). Control cells remained in MEM-10% FBS.

The following antibodies were used for immunofluorescence: mouse monoclonal anti-αSMA clone 1A4 (Sigma-Aldrich, St Louis, MO); rabbit polyclonal anti-GFP (Santa Cruz); mouse monoclonal anti-HA 16B12 (Covance, Princeton, New Jersey); AlexaFluor 488 goat anti-mouse, AlexaFluor 568 goat anti-rabbit (Invitrogen, Carlsbad, CA).

The following antibodies were used for western blot analysis: mouse monoclonal anti-active-β-catenin clone 8E7 (ABC) (Millipore, Billerica, MA); mouse monoclonal anti-β-catenin clone 14 (BD Transduction, San Jose, CA); rabbit polyclonal anti-GAPDH (Santa Cruz, Santa Cruz, CA); goat anti-mouse, anti-rabbit, and rabbit anti-goat IgG-HRP secondary antibodies (Bio-Rad, Hercules, CA).

Immunohistochemistry- Lung tissue was obtained from three patients with SSc-associated pulmonary fibrosis who were undergoing lung transplant surgery

and from three normal donors whose lungs were not used for transplantation. The experimental protocol was approved by the University of Pittsburgh Institutional Review Board. Immunohistochemistry was performed using a monoclonal antibody to β-catenin (BD Biosciences, Cat. 610153, 1:100) and secondary antibodies (Dako Cytomation Envision + System-HRP (DAB)). Imaging was performed using Zeiss Axioskop with Cri Nuance multispectral camera.

Transfections and β-catenin/TCF Reporter Assay- NLHFs were transfected with a TCF Optimal luciferase Promoter plasmid (TOPflash; containing 4 consensus TCF-binding sites upstream of a minimal c-fos promoter) or FOPflash (containing 4 mutant TCF-binding sites; kindly provided by H. Clevers, Utrecht, Netherlands), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). A thymidine kinase (TK)-Renilla plasmid (0.1μg) was also included to normalize luciferase values to the efficiency of transfection. Cells were solubilized 2 days after transfection using the Dual-Luciferase Assay Kit (Promega, Madison, WI), and luciferase activity was quantified using a microplate dual-injector luminometer (Veritas, Sunnyvale, CA). Each reporter plasmid/condition was transfected into cells plated in triplicate wells, and data were expressed as the mean ± standard deviation (SD).

Ki67 Cell Proliferation Assay- Fibroblasts were seeded onto sterile 18mm round cover slips in a 12-well culture plate (approximately 1 x 10^5 cells/well) with serum-free medium. Cells were infected in triplicate with the indicated adenovirus in serum-containing medium 24 hours later. Ad-CMV-GFP was used to equalize total viral load. After incubation for an additional 24 hours, cells were returned to serum-free media for an additional 24 hours, then rinsed in PBS and fixed in 4% paraformaldehyde-PBS for 30 minutes at room temperature. Then the cells were blocked for 30 minutes in 10% normal goat serum (NGS) in PBS before incubation with mouse monoclonal anti-Ki67 (DAKO MIB-1, 1:100) for 1 hour, then washed and incubated in Alexa Fluor 568 goat-anti-mouse IgG (Molecular Probes,1:200) for 30 minutes. Finally cells were incubated with Hoechst 33342 (Invitrogen, 1:10000), rinsed, and mounted with Aqua Poly/Mount (Polysciences, Inc.). Ki67-positive (red) cells and blue nuclei (total cells) were counted on 10 fields per slip using a 63X Plan-Neofluar NA 1.25 objective and Zeiss Axioplan 2 microscope (Carl Zeiss, Inc.).

Quantification of mRNA- Total RNA was extracted from NHLF using RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to manufacturer instructions. First strand cDNA was reverse transcribed from equal amounts of total RNA using iScript cDNA synthesis kit (Biorad, Hercules, CA). Human GAPDH forward primer 5'-GGTGAAGGTCGGAGTCAACG and reverse primer 5'-GGATTTCCATTGATGACAAGCTTC ; human AXIN2 forward primer 5'-ACAACAGCATTGTCTCCAAGCAG

C and reverse primer 5'-GCGCCTGGTCAAACATGATGGAAT; human C-MYC forward primer 5'-TGCGACGAGGAGGAGAACTT and reverse primer 5'-TGGGCAGCAGCTCGAATTTC; human CYCLIND1 forward primer 5'-TGCTCCTGGTGAACAAGCTCAAGT and reverse primer 5'-CGCGTGTTTGCGGATGATCTGTTT; human MMP1 forward primer 5' CAAGATTTCCTCCAGGTCCA and reverse primer 5'-AGGTCTCTGAGGGTCAAGCA; human MMP2 forward primer 5'-CCTGTTTGTGCTGAAGGA and reverse primer 5'-CAAGAAGGGGAACTTGCA. Real-time PCR (qPCR) was performed using iQ SYBR Green Supermix according to the manufacturer instructions (Biorad, Hercules, CA). PCR was carried out in 96-tube plates using the MyiQ Single Color Real-Time PCR Detection System and software (Bio-Rad). All reactions were performed in triplicate with negative controls. The results represent the mean ± SD from three independent experiments. GAPDH was used as the internal control. The relative change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method(2).

Collagen Contraction Assay- Type I collagen gels were prepared by mixing rat tail HC collagen (BD Biosciences) with 10x Hanks (Sigma-Aldrich, St Louis, MO) and 7.5% $NaHCO_3$ to final concentration of 1 mg/ml. After 24 hours of infection with the specified adenovirus with and without TGF-β (5 ng/ml), NHLFs were added to the collagen mixture (final concentration of 3.0 $x10^5$ cells/ml and 0.75 mg/ml collagen). After aliquots were cast into 24-well plates, the diameter of each collagen matrix was measured at 0, 24, 48, 72 and 96 hours. The results were expressed as percentage contraction [(Initial-End diameter)/Initial diameter x 100%].

Cell Migration Assay- Cells were grown as monolayer to 80% confluence and a 200 μl pipette tip was used to create a linear scratch. Cells were then infected with the indicated adenovirus in medium containing 10 μg/ml of Mitomycin C. Wound surface area was quantified from captured images using Metamorph analysis software (Molecular Devices). For Transwell migration, adenovirus-infected cells were seeded on the upper chamber of 6-well membrane inserts (3.0 μm pore size, Becton Dickinson, CA). Medium containing 20 μg/ml of human plasma fibronectin (Invitrogen, CA) filled the lower chamber as a chemoattractant. After incubation for <22 hours (less than one doubling for NHLFs), cells on the upper side of the membrane were removed with a swab and cells on the reverse side of the membrane from four low magnification fields were counted for each membrane. Data are expressed as mean ± SD.

References

1. Pilewski JM, Liu L, Henry AC, Knauer AV, Feghali-Bostwick CA. Insulin-like growth factor binding proteins 3 and 5 are overexpressed in idiopathic pulmonary fibrosis and contribute to extracellular matrix deposition. *Am J Pathol* 2005;166(2):399-407.

2. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods* 2001;25(4):402-408.

3. Maher MT, Flozak AS, Stocker AM, Chenn A, Gottardi CJ. Activity of the beta-catenin phosphodestruction complex at cell-cell contacts is enhanced by cadherin-based adhesion. *J Cell Biol* 2009;186(2):219-228.

Figure Legends

Supplemental Figure 1: Activation of β-catenin/TCF signaling in NHLFs by adenoviruses expressing Wnt3a and S37A-βcatenin shows increased signaling and stabilization of β-catenin. (A) NHLFs were infected with Ad-GFP, Ad-Wnt3a and Ad-S37A-β-catenin at increasing PFU/cell and simultaneously transfected with the β-catenin/TCF reporter plasmid, TOPflash (FOPflash control, see Methods). (B and C) NHLFs infected with Ad-GFP, Ad-Wnt3a and Ad-S37A-β-catenin at increasing PFU/cell show an increase in the N-terminally unphosphorylated, transcriptionally Active form of β-Catenin (ABC) by immunoblot analysis at 48 hours post-infection. An increase in the cytosolic signaling pool of β-catenin can be also shown by affinity precipitation with recombinant ICAT (GST-ICAT) 48 hours post-infection, as described in (3).

Supplemental Figure 2: Overexpression of non-degradable β-catenin increases lung fibroblast proliferation independent of altered c-myc or cyclin D1 expression. (A) NHLFs were infected with Ad-GFP, Ad-S37A-β-catenin, Ad-ICAT, and Ad-Wnt3a for 48 hours and then pulse-labeled with BrdU as described in Methods. Results are expressed as mean ± SD relative to control adenovirus from at least 3 independent experiments (** $p<0.01$). (B) Representative images of Ki67 immunostaining in NHLFs infected with the indicated adenoviruses. Quantification of the Ki67-positive cells shown in Figure 5B. (C and D) NHLFs infected with adenovirus expressing S37A-β-catenin or Wnt3a for 24 hours do not show significant upregulation of c-myc (C) or cyclin D1 (D) gene expression by real-time PCR.

Supplemental Figure 3: Overexpression of Wnt3a and non-degradable β-catenin stimulates fibroblast migration independent of altered MMP-1 or MMP-2 expression.

(A) NHLFs were infected with Ad-GFP, Ad-S37A-β-catenin, Ad-ICAT, and Ad-Wnt3a and analyzed in a Boyden chamber assay. Results are expressed as the mean fold change in each condition relative to control adenovirus ± SD from 3 independent experiments (*p<0.05). (B and C) NHLFs infected with adenovirus expressing S37A-β-catenin or Wnt3a for 24 hours do not show substantial upregulation of MMP-1 (B) or MMP-2 (C) gene expression by real-time PCR, despite clear effects on motility (Figure 6).

Supplemental Figure 4: Normal adult human lung fibroblasts are responsive to TGF-β. NHLFs were treated with TGF-β (5 ng/ml) for 48 hours, immunostained for α-SMA (A) and quantified (B).