APPENDIX AVAILABLE ON THE HEI WEB SITE

Research Report 182

Synergistic Effects of Particulate Matter and Substrate Stiffness on Epithelial-to-Mesenchymal Transition

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Appendix A. The Role of Mechanics in Particle Uptake

Note: Appendices available only on the Web have been reviewed solely for spelling, grammar, and cross-references to the main text. They have not been formatted or fully edited by HEI.

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This document was reviewed by the HEI Health Review Committee.

Appendix A. The Role of Mechanics in Particle Uptake

Our preliminary work proposed looking at how mechanics affected particulate endocytosis. Upon completion of several preliminary experiments with inconclusive results, we reformulated our hypotheses and investigated the breadth of research presented in the accompanying report. Our findings from these preliminary experiments are presented below.

INTRODUCTION

Substrate stiffness has recently been shown to enhance nonviral gene delivery through enhanced actin polymerization and subsequent endocytosis of DNA-containing particulates (Kong et al. 2005). In addition, the activation of $\alpha 5\beta 1$ integrin via fibronectin binding has been shown to enhance DNA particulate and bacterial endocytosis (Agerer et al. 2005) and has been linked to $\beta 1$ integrin recycling (Proux-Gillardeaux et al. 2005). Functionally blocking $\beta 1$ integrin with antibodies inhibits endocytosis of particles (Ozeri et al. 1998), presumably through the inhibition of the Src family kinase substrate cytoskeletal adapter protein cortactin (Agerer et al. 2005), a protein pivotal in endocytosis (Fowler et al. 2003). Src family kinases also mediate the activation states of the Rho family of small GTPases, specifically the small GTPase Rac1, which has been significantly linked to particle endocytosis (Morehead et al. 2002).

Interestingly, the addition of fibronectin has been shown to potently increase cellular uptake of retroviruses (Hanenberg et al. 1997) and polycationic microparticles, presumably through the activation of the Src-Rac1 endocytotic pathway. We believe that fibronectin exists in multiple structural states at the molecular and matrix level that affect integrin specificity and in turn the activation of critical endocytotic pathways. Thus individuals with aberrant fibronectin assembly and organization, as is the case in pulmonary fibrosis, might be predisposed to enhanced PM internalization. In this work we investigated if increased substrate stiffness affects particle endocytosis.

METHODS

Expression and purification of Fn fragments

Cloning and expression of recombinant proteins – Complimentary cDNAs encoding the 9th and 10th Fn type III repeats (III9-10) with a Fc linker were amplified from a human cDNA library (ATCC, Manassas, VA) by the use of appropriate oligonucleotide primers (Invitrogen, Carlsbad, CA) in polymerase chain reactions with Pfu polymerase (Stratagene, La Jolla, CA). The amplified cDNA products were then inserted into pGEX4T-1 (GE Healthcare) to generate glutathione S-transferase (GST) fusion products with a spanning thrombin-sensitive cleavage site. The Fn fragment mutants (III9*10, III9p10, III92G10, and III94G10) were made using the Quickchange protocol (Stratagene). All sequences were confirmed prior to transformation for protein production.

The expression vectors were then inserted into BL21 *E.coli* by electroporation at 25 µF, 2.5 kV, and 2000 ohms for 4.5–5 milliseconds. The clones that produced the highest level of protein were expanded, and recombinant protein expression was induced by IPTG. The cell pellets were resuspended in ice-cold PBS supplemented with protease inhibitors and 10 U/mL DNAse I. The cells were lysed by adding 1mg/mL lysozyme and then sonicated. The cell lysate was then cleared of cellular debris by centrifugation and filtration through a 0.22-µm pore filter.

Recombinant Protein Purification – GST-fusion proteins was purified using a AKTAFPLC (GE Healthcare) with a GSTPrep FF 16/10 column (GE Healthcare) following standard procedures. The recombinant fusion protein binds the glutathione (GSH)-coupled resin in PBS, pH 7.4, and can be eluted with reduced GSH (50 mM Tris-HCl, 10 mM reduced GSH, pH 8.0). The purified recombinant protein was then incubated overnight with thrombin (Sigma-Aldrich, St. Louis, MO) to remove the GST tag. Following the removal of the excess soluble GSH, the cleaved recombinant protein was reintroduced on the GSTPrep FF 16/10 column to remove the soluble GST followed by a HiTrap Benzamidine FF column (GE Healthcare) for removing

thrombin. The recombinant proteins were concentrated with a 10-kDa Amicon Ultra centrifugal filter device and the buffer exchanged to TBS (18 mM Trizma hydrochloride, 135 mM NaCl, pH 7.4). The protein solutions were filter-sterilized using a 0.22-µm filter syringe filter and verified as >95% pure by SDS PAGE. Finally, endotoxin levels were verified as under 0.1 EU/mL by the use of the Limulus Amebocyte Lysate (LAL) QCL-1000 kit (East Rutherford, NJ, USA).

Particle Uptake Cell Culture Studies

Primary ATII cells or RLE-6TN cells, an alveolar epithelial cell line, were grown in DMEM/F12 media supplemented with 10% FBS and 1% penicillin/streptomycin. To determine the effect of substrate stiffness on epithelial cell particle uptake, cells were plated on polyacrylamide gels with the specified ECM protein cross-linked on the surface with rigidities in the following range: E = 4–32 kPa, or on glass coverslips with fibronectin or laminin as positive and negative controls, respectively.

ATII Cell Particle Internalization

RLE-6TN (ATII) cells were cultured on the PA gels of various stiffnesses in DMEM/F12 media with 10% fetal bovine serum (FBS). Fn (45 nM) and Ln (45 µM) coated 25-mm glass coverslips were used as controls in all experiments. In a two-day experiment, the cells were seeded at 50,000 cells/cm^2 on the PA gels and allowed to grow and attach overnight (16 hours) before addition of fluorescent microparticles. In a one-day experiment, the cells were seeded on the PA gels and allowed to grow and attach for 4 hours before addition of fluorescent microparticles.

After the ATII cells were attached to the PA gels, 2-µm yellow-green fluorescent (excitation 488 nm, emission 505/515 nm) sulfate FluoSpheres® beads (Invitrogen/Molecular Probes) were added to the cells on the gels (4, 8, 16, 24, 32 kPa) in 6 well plates at a concentration of either 1 million or 10 million beads per mL. The 2-µm-diameter polystyrene sulfate beads were chosen

for their relatively hydrophobic properties that allow for passive adsorption of almost any protein, and their diameter reflected the typically most harmful size of particulate matter ($PM_{2.5}$). Cells were incubated with the particles for 4 hours, determined by a particle internalization kinetic study to understand the optimal time for particle endocytosis before flow cytometry analysis.

Flow cytometry analysis of RLE-6TN cells with internalized fluorescent microparticles

After incubating with the fluorescent microparticles for 4 hours, cells were washed three times with PBS to remove any non-internalized particles from the cells and media. 0.25% Trypsin + EDTA was added to the cells on the gels for 5 minutes to detach the cells from the substrates and glass coverslips. Additional tapping of the wells was necessary after Trypsin/EDTA incubation to ensure cellular detachment from the PA gels. The cells were then quenched with 10%-FBS-containing media and pooled into microcentrifuge tubes to concentrate the samples for flow cytometry. After centrifugation, the cells were resuspended in smaller volumes of cell culture media and filtered through 20-µm mesh cell strainers into flow cytometry tubes. The tubes were placed on ice and run on a C6 Accuri Flow Cytometer (Accuri Cytometers, Ann Arbor, MI) for analysis on the FL1-A channel (488-nm excitation/530-nm±15-nm emission).

Previous initial studies were also performed to determine and characterize the fluorescence of RLE-6TN cells and individual fluorescent microspheres. Cells were identified by their forward:side scatter profiles, and fluorescence measurements made on at least 10,000 cells per condition. Typical FSC:SSC plots are presented as are representative FITC histograms of the cell population.

RESULTS

Particle internalization inhibition with sodium azide

In order to verify that particle uptake by the ATII cells was an energy-dependent process, RLE-6TN cells were plated at 50,000 cells/cm^2 on tissue culture polystyrene and allowed to grow and attach overnight. Four hours before the addition of microparticles, several concentrations (10 mM and 100 mM) of sodium azide (NaN_3) were added to the cell culture media in order to deplete cellular ATP. 10 million particles/mL of 2-µm sulfate FluoSpheres fluorescent microspheres were added to the cells for 4 hours. Afterwards, the cells were washed and prepped for flow cytometry analysis. Our analysis shows that with increasing concentrations of sodium azide, the percentage of cells with internalized particles decreased dramatically. This validated our hypothesis that the cells would require energy in order to internalize the particles (Appendix Figure A.1).

ATII cells cultured on increasingly stiff substrates (within the physiologic range) do not show enhanced particle endocytosis in vitro

The primary goal of this study was to explore the role of tissue stiffness in particle endocytosis. To explore this relationship, we proposed to use polyacrylamide gels to generate substrates of increasing stiffness. As a control we used glass coverslips or tissue culture plastic coated with Fn or Ln. ATII cells were seeded on the substrates, allowed to attach and spread overnight (~16 hours), and then treated with 2-µm fluorescent polystyrene particles. Particle endocytosis was quantified by flow cytometry (Appendix Figure A.2). This analysis enables not only the determination of the percent total cell population containing particles (i.e., uptake of one or more particles) but also, because of the very well-defined nature of the particles, the determination of the number of particles each cell internalized. ATII cells cultured on 2-kPa gels did not consistently spread within the short timing of the experiment, and so these data are inconclusive. As seen in Appendix Figure A.3, flow cytometry analysis indicates that there are

distinct populations of ATII cells that take up 0, 1, 2, 3, and more than 4 particles. From the flow histogram these populations can be specifically segregated and quantified. Based on experiments such as those represented in Appendix Figure A.4, we were unable to observe a gross trend in substrate stiffness affecting particle internalization.

Differential ATII cell integrin engagement does not affect particle internalization

ATII cell integrin engagement on different fibronectin fragments might mimic cells adhering to tissues of different stiffness with different degrees of Fn unfolding. We hypothesized that this might affect the ability of these cells to internalize particles. In a similar experiment as described above, cells were plated on tissue culture plastic with immobilized Fn fragments, Fn, or Ln. Cells were allowed to attach and spread overnight (~16 hours). FITC-labeled polystyrene particle were added to the culture media and incubated with cells for 4 hours. Particle uptake was analyzed by flow cytometry (Appendix Figure A.5). Two independent triplicate experiments indicate that 30% to 50% of ATII cells take up particles regardless of the underlying substrate. A greater percentage of cells on Fn and Ln are positive for particles, compared with the two Fn-fragment variants, but these increases are not statistically significant. There are no observable differences in particle uptake between cells cultured on either Fn-fragment variant.

Inhibition of ATII cell contraction increase particle internalization

In order to determine how cell contractility affects particle internalization, ATII cells were seeded on the various Fn-fragment substrates and allowed to attach and spread overnight (~16 hours). Y-27632 (a pharmacologic cell-contractility inhibitor) was added to a final concentration of 10 µM to the cell culture media 1 hour before particle addition. One million particles/mL were added and incubated with the cells for 4 hours. The cells were then washed 3 times with PBS, trypsinized, and resuspended for flow cytometry analysis. Interestingly, inhibiting Rho-associated kinase (ROCK) with the pharmacologic inhibitor Y-27632 induced an increase in

particle uptake (Appendix Figures A.5 and A.6). These data suggest that increased Rho signaling suppresses, rather than augments, particle uptake in ATII cells.

Overall, our data show that engaging various molecular variants of Fn or engaging substrates of various stiffnesses has no effect on particulate endocytosis. However, the process of particle endocytosis might be potentially mediated by Rac/CDC42 and not Rho, as internalization increases with ROCK inhibition.

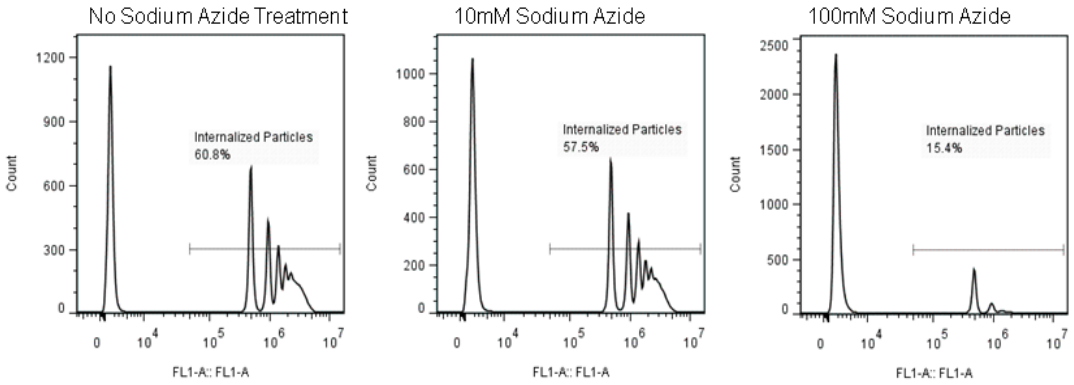

Figure A.1. ATII cell particle uptake is inhibited by sodium azide (NaN_3) treatment.

Treatment of cells with the ATP-pump inhibitor NaN_3 blocks all particle uptake, suggesting that the process is energy-dependent. Fewer particles are internalized with increasing concentrations of NaN_3.

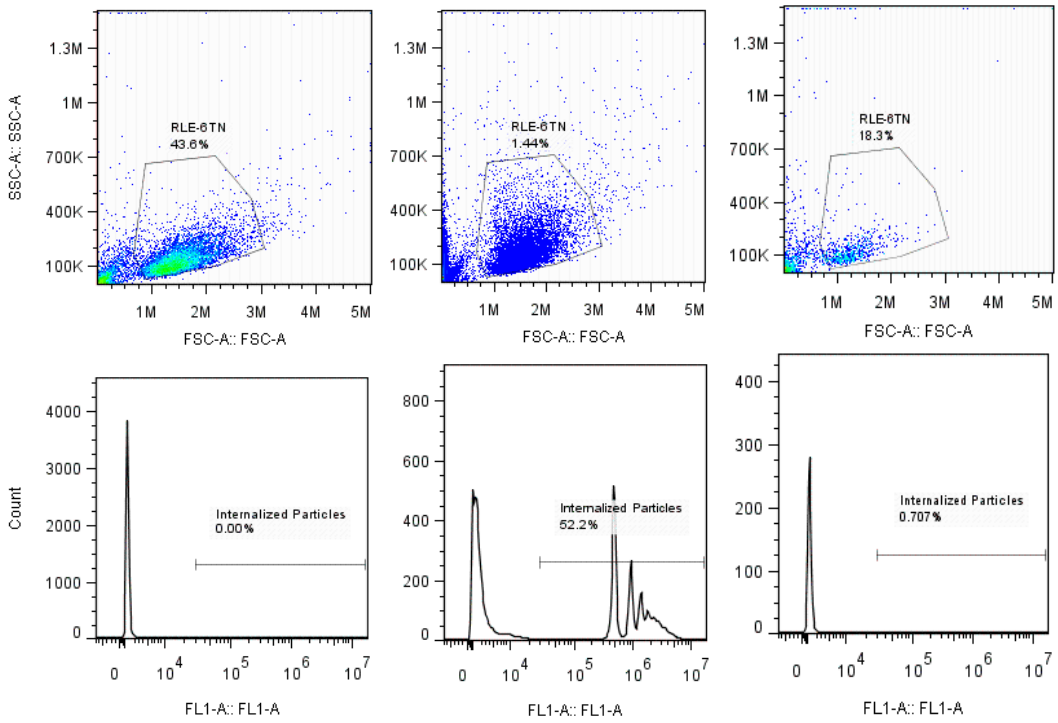

Figure A.2. Flow cytometry analysis of ATII cell particle internalization. (Top) ATII cell FSC:SSC and (bottom) FITC histogram plots. (Left) Untreated cells, (middle) particle-treated cells, and (right) particle + NaN_3 treated on 16-kPa substrates (representative). The side scatter (SSC) of the cells increases in the particle-treated group because of the increased scattering caused by internalized particles. (Bottom left) Untreated cell background fluorescence helps establish the gating. (Bottom right) Particle-treated cells are shifted strongly to the right, indicative of internalized FITC particles. Cells with 1, 2, 3, and > 4 particles can easily be distinguished. Treatment of cells with the ATP-pump inhibitor NaN_3 blocks all particle uptake, suggesting the process is energy-dependent.

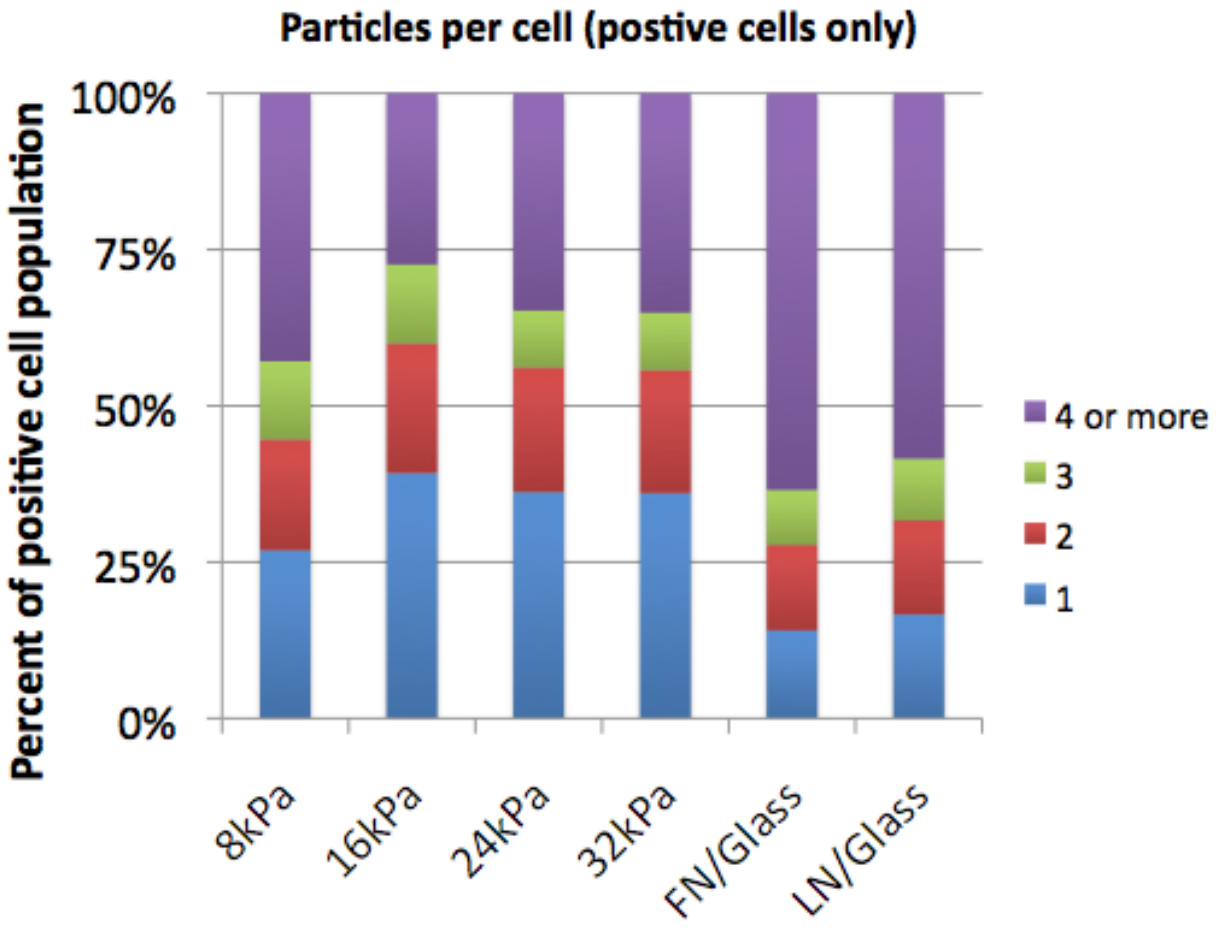

Figure A.3. ATII cell breakdown of the positive population – number of particles internalized. Flow cytometry histograms were gated to determine the percentage of the positive population that had internalized each number of particles. The only observable differences are the increase in the number of cells that internalize > 4 particles and the decrease in the number of cells that internalize only 1 particle on glass substrates (GPa stiffness).

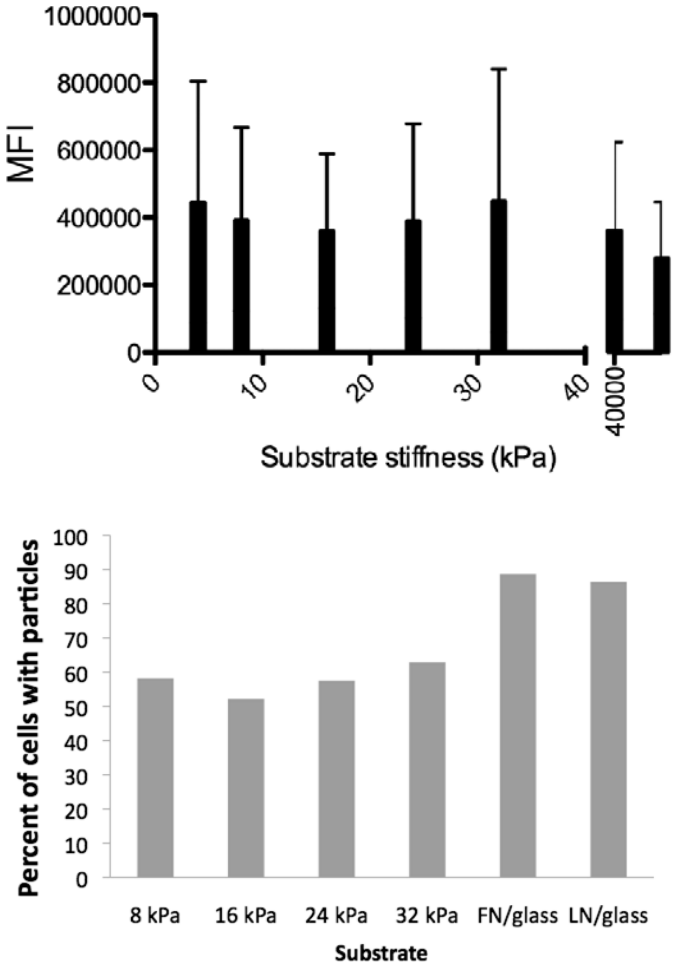

Figure A.4. Flow cytometry mean fluorescence intensity (MFI) and percentage of ATII cells of the total population with internalized particles show no significant trend with substrate stiffness. (Top) MFI of ATII cells incubated with FITC particles on increasingly rigid substrates. Far right columns represent Fn- and Ln-coated glass, respectively. (Bottom) MFI data, represented as a percentage of the total ATII cell population positive for particle uptake. All positive cells are counted regardless of the number of particles in the cell (i.e., one or more particles per cell).

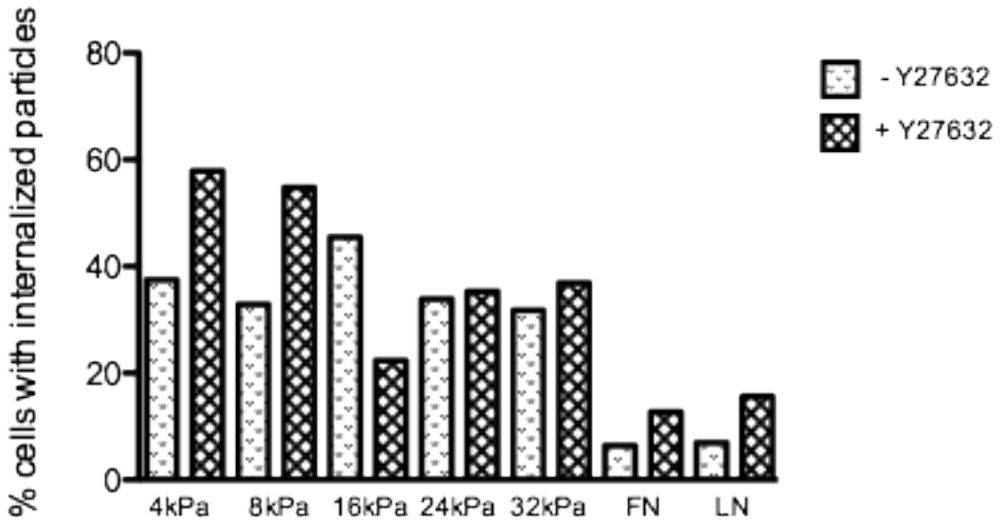

Figure A.5. ROCK inhibition in general increases the percentage of ATII cells with internalized particles. Percent of ATII cell populations with internalized particles +/- the ROCK inhibitor Y-27632 is shown above. Cells were allowed to grow and spread overnight on fibronectin-coated substrates of various degrees of stiffness. Far right columns represent Fn- and Ln-coated glass, respectively. FITC particles were incubated with the cells for 4 hours the next morning before flow cytometry analysis. ROCK-treated groups were treated with 10 µM of the inhibitor for 1 hour before particle exposure. All positive cells are counted regardless of the number of particles in the cell (i.e., 1 or more particles per cell).

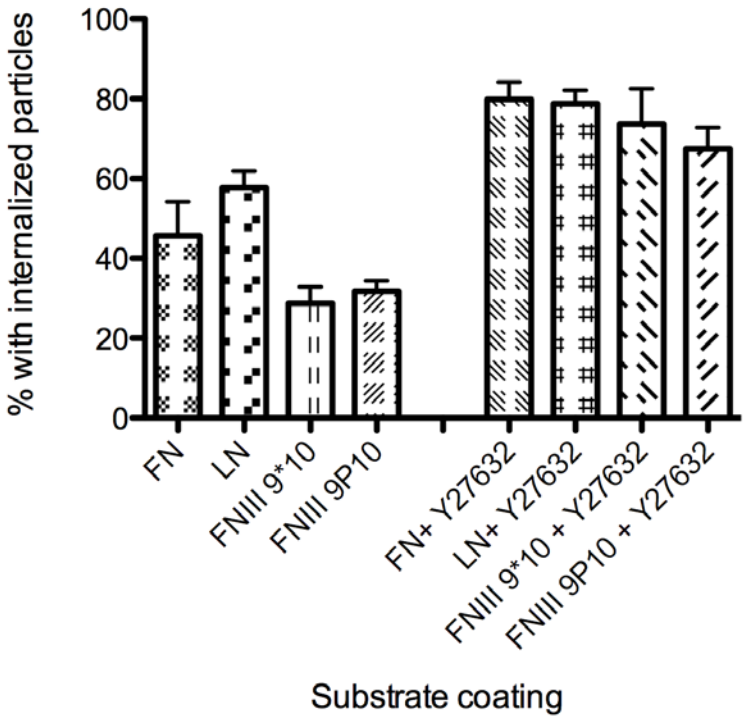

Figure A.6. ATII cell particle uptake is not influenced by Fn fragments that support integrin selective adhesion and spreading. ATII cells seeded on Fn, Ln, FnIII9*10, or FnIII9P10 show similar levels of particle uptake by flow cytometric analysis. The inhibition of ROCK with Y-276332 further augments particle uptake.