Fibronectin on the Surface of Extracellular Vesicles Mediates Fibroblast Invasion

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Abstract

Extracellular vesicles (EVs) are endosome and plasma membrane-derived nano-sized vesicles that participate in intercellular signaling. Although EV cargo may signal via multiple mechanisms, how signaling components on the surface of EVs mediate cellular signaling is less well understood. In this study, we show that fibroblast-derived EVs carry fibronectin on the vesicular surface, as evidenced by mass spectrometry-based proteomics (Sequential Window Acquisition of all Theoretical Mass Spectra) and flow-cytometric analyses. Fibroblasts undergoing replicative senescence or transforming growth factor β 1-induced senescence and fibroblasts isolated from human subjects with an age-related lung disorder, idiopathic pulmonary fibrosis, secreted higher numbers of EVs than their respective controls. Fibroblast-derived EVs induced an invasive phenotype in recipient fibroblasts. This invasive fibroblast phenotype was dependent on EV surface localization of fibronectin, interaction with the fibronectin receptor α 5 β 1 integrin, and activation of invasion-associated signaling pathways involving focal adhesion kinase and Src family kinases. EVs in the cellular supernatant, unbound to the extracellular matrix, were

capable of mediating invasion signaling on recipient fibroblasts, supporting a direct interaction of EV surface fibronectin with the plasma membrane of recipient cells. Together, these studies uncover a novel mechanism of EV signaling of fibroblast invasion that may be relevant in the pathogenesis of fibrotic diseases and cancer.

Keywords: extracellular vesicles; fibroblast; fibronectin; idiopathic pulmonary fibrosis; invasion

Clinical Relevance

Extracellular vesicles (EVs) mediate cell-cell communication, although the underlying mechanisms are not well understood. Here, we report that senescent cells secrete higher numbers of EVs that carry fibronectin on their surface. EV surface-associated fibronectin engages integrin receptors on target cells to signal fibroblast invasion, an emerging phenotype of fibrotic lung diseases.

Extracellular vesicles (EVs) are lipid bilayered membrane-derived vesicles that range from 30 to 1,000 nm in diameter and participate in cell-cell communication. EVs

are mainly formed during endocytosis by inward budding of multivesicular endosomes and are released extracellularly after fusion of multivesicular endosomes

with the plasma membrane (exosomes), or by direct shedding of the plasma membrane (microvesicles). These vesicles carry cell-specific cargo that may include biologically

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active DNA, RNA, proteins and lipids that regulate the behavior of recipient cells. EVs dock on recipient cells and deliver cargo either by direct fusion with the plasma membrane or by endocytosis (1, 2). Presence of EVs has been observed within the extracellular matrix (ECM) of both mineralizing and soft tissues (3). EVs have been shown to adhere to the ECM, and to express specific components of the ECM on the vesicular surface (4, 5). The functional significance of EV binding to the ECM, and the expression of ECM components on the vesicle itself are not well defined.

Cellular senescence and altered intercellular communication play critical roles in age-related diseases (6, 7). Fibrotic diseases involving diverse organ systems, including the lung, have been linked to aging. Fibroblast invasion has been proposed as a major driver of disease pathogenesis in lung fibrosis, in particular idiopathic pulmonary fibrosis (IPF) (8, 9). However, the precise mechanisms by which fibroblasts acquire an invasive phenotype are not known. EVs have been recently shown as novel mediators of cellular invasion in wound healing and cancer. EVs isolated from diabetic wound were shown to promote invasion of normal fibroblasts (10), while uptake of exosomes by Kupffer cells in the liver resulted in activation of fibrotic pathways and initiation of a premetastatic niche (11). Whether paracrine effects of EVs drive the invasive phenotype of lung fibroblasts is not known. In this study, we characterized EVs secreted from senescent and nonsenescent lung fibroblasts, and explored their functional role in promoting a profibrotic, invasive phenotype.

Methods

Cell Lines

IMR90 human fetal lung diploid fibroblasts with a very low population doubling level (PDL) were purchased from the Coriell Institute. A cellular model of replicative senescence was established with IMR90 fibroblasts. Those with a PDL between 45 and 55 were termed high PDL (HPDL; senescent), and those with a PDL of <30 were categorized as low PDL (LPDL; nonsenescent) fibroblasts (12). IPF and non-IPF fibroblasts were isolated from the lungs of patients through the University of

Alabama at Birmingham (UAB) Airway Tissue Procurement Program following institutional review board approval. Details regarding the methods used in this work are provided in the data supplement.

Isolation of EVs

EVs were isolated by differential centrifugation. Protocol for the isolation of EVs from cell culture medium, and ECM is provided in the data supplement (13). Size and number of purified vesicles were determined by Nanoparticle Tracking Analysis (NTA) using NanoSight NS300. Data acquisition and analysis was performed using NTA v2.3 software. Five video frames of 60 seconds at camera level 10 from each sample were recorded. The detection threshold was set at 10 to analyze the size and number of the EVs.

Electron Microscopy

An undiluted suspension (10 μ l) of lung fibroblast-derived EVs were negatively stained with osmium tetroxide and imaged on a FEI Tecnai T12 electron microscope at the UAB High Resolution Imaging Facility. The EVs were imaged at 104,000 $\times$ magnification (Figure E1C).

Fibroblast Invasion Assay

Matrigel (BD Biosciences) was mixed in a 1:1 ratio with serum-free medium containing EVs. Invasion of lung fibroblasts through the Matrigel in Transwell chambers was studied according to a standard protocol (14).

EV Protein Analysis by Tandem Mass Spectrometry (MS/MS^{ALL}) with Sequential Window Acquisition of All Theoretical Mass Spectra

EV protein lysates were separated on a 4–20% Tris-glycine Bio-Rad Criterion precast gradient gel under reduced condition, stained with Coomassie R250 stain (Millipore-SIGMA), and subjected to proteome analysis by Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS) (SCIEX).

Flow Cytometry and ImageStream Analysis

A total of 1×10^7 particles of fibroblast-derived EVs were stained in a final volume of 100 μ l for 30 minutes at 4°C with fluorescently labeled antibodies, CD63 eFluor 450 (EV marker; clone H5C6) and

fibronectin-phycoerythrin (FN-PE). Flow cytometry data acquisition was performed on a BD LSRII (BD Biosciences). The acquired flow cytometry data were analyzed using FlowJo X. For ImageStream analysis (Amnis, EMD Millipore), fluorescently labeled (CD63 eFluor 450 and FN-PE) EVs were stained in a final volume of 50 μ l for 30 minutes at 4°C. The acquired data were then analyzed using the IDEAS software.

Western Blotting

Western blotting was performed as previously described (15).

Immunofluorescence Labeling of EVs

Fibroblast-derived EVs were fluorescently labeled with Exo-Red (System Biosciences; acridine orange, which selectively stains nucleic acid) according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses and graphical presentations were prepared in GraphPad PRISM version 7.0 software. Western blotting and NanoSight particle tracking data obtained in this study were analyzed by unpaired two-tailed Student's *t*-test for pairwise comparisons. If more than two groups were present, the data were analyzed by ANOVA followed by Tukey's *post hoc* test for multiple pairwise comparisons. Results were considered significant if $P < 0.05$.

Results

Characterization of EVs Secreted by Nonsenescent and Senescent Lung Fibroblasts

Cellular senescence has previously been associated with diseases of aging, including IPF; however, the role of EVs in fibroblast senescence and fibrogenesis is not well understood. We purified and analyzed EVs released by nonsenescent fibroblasts (defined as LPDL) and senescent fibroblasts (defined as HPDL; see METHODS for details). EVs were analyzed for number and size by nanoparticle tracking (NanoSight NS300). All EV counts were normalized to the total cell number and adjusted for the dilution factor. Senescent fibroblasts released a significantly higher number of EVs (represented as EV count/cell/ml) than nonsenescent fibroblasts. There was no

significant difference in EV numbers in the culture media between senescent and nonsenescent fibroblasts, whereas the ECM

of senescent fibroblasts contained a significantly higher number of EVs than that of nonsenescent fibroblasts (Figure 1A;

representative experiment). Similar patterns were observed in additional experiments (Figures E2A and E2B). To ensure that recovery of EVs was similar in HPDL and LPDL fibroblasts, the known numbers of EVs isolated from LPDL and HPDL fibroblasts were reprecipitated by ultracentrifugation. The results indicated a significant loss of EVs after reprecipitation in both EV populations. Interestingly, this loss was significantly greater ($P < 0.001$) in HPDL-derived EVs than in LPDL-derived EVs (Figure E1D). This suggests that there was a difference in sedimentation rate between these two EV populations, and further strengthens our finding that senescent (HPDL) fibroblasts released significantly higher numbers of EVs than nonsenescent (LPDL) fibroblasts despite the lower sedimentation rate of HPDL-derived EVs.

There was no significant difference in EV size between senescent and nonsenescent fibroblasts isolated from either culture medium (Figure 1B). Although the majority of isolated EVs from both senescent and nonsenescent fibroblasts were less than 200 nm in diameter, consistent with exosomes, a smaller fraction of isolated EVs were up to 400 nm in diameter. We suspect that these larger particles represent either exosomal aggregates or plasma membrane-derived microvesicles. We observed no significant difference in the degree of heterogeneity of particle size between EVs purified from either culture medium or ECM (Figures E1A and E1B).

Transforming growth factor $\beta 1$ (TGF- $\beta 1$) is a known fibrogenic mediator (15) and mediates senescence-like effects on fibroblasts (16); however, its effects on EVs are unknown. We found that, similarly to senescent fibroblasts, TGF- $\beta 1$ stimulated nonsenescent fibroblasts to release a higher number of EVs, which were primarily bound to the ECM (Figure 1C). No significant difference in the size of the EVs before and after TGF- $\beta 1$ treatment was observed (Figure 1D). Accumulation of senescent fibroblasts has been reported in IPF (7, 16). We characterized EVs released by fibroblasts isolated from lungs of human subjects with IPF (IPF fibroblasts) and healthy control subjects (non-IPF fibroblasts). IPF fibroblasts released significantly higher numbers of EVs than non-IPF fibroblasts (Figure 1E). Similar results were observed in two additional non-IPF and IPF cell lines (Figures E2C and E2D). No significant difference in EV sizes

Figure 1. Characterization of extracellular vesicles (EVs) secreted by lung fibroblasts. EVs were isolated from the culture medium (CM) and extracellular matrix (ECM) of nonsenescent (low population doubling level [LPDL]) and senescent (high PDL [HPDL]) lung fibroblasts by differential centrifugation. (A and B) The number and size of the EVs were determined by nanoparticle tracking analysis using NanoSight NS300 and NTA 2.3 analytical software. A representative experiment is shown; similar patterns in the relative numbers of EVs were observed in two additional separate experiments (Figures E2A and E2B). Nonsenescent lung fibroblasts were serum deprived overnight and treated with either vehicle or TGF- $\beta 1$ (2.5 ng/ml) for 48 hours. (C and D) EVs were purified from the culture medium and ECM, and analyzed for number and size. (E and F) EVs were also purified from the culture medium and ECM of non-IPF and IPF lung fibroblasts and compared for number and size. The total number of EVs counted per milliliter were adjusted for the dilution factor and normalized to the total number of fibroblasts. A representative cell line is shown; similar patterns in the relative numbers of EVs were observed in two additional non-IPF and IPF cell lines (Figures E2C and E2D). Data are mean $\pm$ SEM, $n = 5$ technical replicates; * $P < 0.0001$. The EV sizes shown here represent pooled data from three separate experiments. IPF = idiopathic pulmonary fibrosis; TGF- $\beta 1$ = transforming growth factor- $\beta 1$.

was observed between non-IPF and IPF EV populations (Figure 1E). Due to heterogeneity among the different experiments and human subject samples, we were not able to “pool” the data into a single graph; however, similar results were observed in multiple experiments at different time points. These data indicate that fibroblasts induced to undergo replicative senescence (senescent vs. nonsenescent), TGF- β 1-induced senescent fibroblasts, and fibroblasts isolated from human subjects with IPF secrete higher numbers of EVs.

Proteomics Analyses of Lung Fibroblast-derived EVs

Next, we determined whether there were qualitative differences in protein expression

in EVs derived from senescent and nonsenescent fibroblasts. EVs from culture media of LPDL cells were analyzed by SWATH-MS for protein/peptide cargo. First, a peptide library was created combining EVs purified from nonsenescent and senescent fibroblasts. A total of 718 unique peptides were detected by SWATH-MS, including the predominant EV markers CD9, CD63, CD81, and HSP70 (Table E1). EV protein lysates were also subjected to Western blotting analysis using antibody against calnexin to exclude any endoplasmic reticulum membrane contamination (Figures E3A and E3B). We noted an abundance of ECM proteins in the EVs, in particular FN, consistent with other studies

(4, 17) (Table E1). When exosomal peptides from nonsenescent fibroblasts were compared with those from senescent fibroblasts, a total of 609 common peptides were identified. Seventy-two peptides were found to be significantly more abundant in nonsenescent EVs, whereas only 17 peptides were significantly more abundant in senescent EVs (Table E2). Gene ontology enrichment analysis of our EV peptide database showed 479 out of 718 peptides that are known to be associated with EVs, confirming the purity of our EV preparations (Table E3). Furthermore, enrichment analysis by pathway mapping indicated roles of EVs in cellular adhesion and ECM remodeling (Table E4).

Figure 2. Matrigel invasion assay. Diagram showing the protocol for EV-mediated fibroblast invasion assay. EVs were seeded in various amounts (0–500 ng) in Matrigel diluted 1:1 with serum-free medium on ice, and allowed to form a gel at 37°C in 8.0- μ m pore size Transwell chambers in a 24-well cell culture dish. Fibroblasts (10^5 cells/100 μ l in serum-free medium) were layered on the top of the Matrigel and allowed to invade overnight against a gradient of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS added in the lower chamber of the Transwell. (A) Invasive cells were stained with crystal violet and imaged. (B) Panel showing EV-mediated invasion of nonsenescent lung fibroblasts through Matrigel. Note that the greater the amount of EVs in the Matrigel, the greater the invasion capacity of the fibroblasts. Scale bars: 100 μ m. (C) The bar graph represents the number of fibroblasts invaded through Matrigel per high-power field ($\times 400$). Data are mean $\pm$ SEM; $n = 3$ technical replicates; five random fields were counted for each replicate; * $P < 0.0001$ versus control. (D) Nonsenescent lung fibroblasts were incubated with nonsenescent lung fibroblast-derived EVs (500 ng) for 1 hour, and the EVs were then removed by centrifugation and subjected to invasion assay through Matrigel without EV seeding. Scale bars: 100 μ m. (E) The bar graph represents the number of fibroblasts invaded through Matrigel per high-power field ($\times 400$). Data are mean $\pm$ SEM; $n = 3$ technical replicates; five random fields were counted for each replicate; * $P < 0.0001$ versus control (no EV).

Figure 3. Flow cytometry analysis to determine fibronectin (FN) expression on the surface of EVs. EVs isolated from the culture medium and ECM of non-senescent lung fibroblasts were either single labeled or dual labeled with antibodies against FN (phycoerythrin labeled) and EV marker CD63 (eFluor 450 labeled). (A) Unstained EVs were used as control for autofluorescence. (B) FN- and CD63-labeled EVs were also analyzed by imaging flow cytometry (ImageStream) for visualization of dual expression on the surface. FSC-A = forward scatter area; SSC-A = side scatter area.

EVs Promote Fibroblast Invasion

To determine whether EVs modify the invasive capacity of lung fibroblasts, EVs purified from fibroblasts were mixed with Matrigel (diluted 1:1 with serum free medium) in varying amounts (0, 10, 100,

and 500 ng) and tested for fibroblast invasion through Matrigel (Figure 2A). Nonsenescent (LPDL) lung fibroblasts were used in all of the invasion assays, as senescent (HPDL) lung fibroblasts did not show invasion with or without EVs

contained in the Matrigel (data not shown). The results indicated there was an EV number-dependent increase in fibroblast invasion (Figures 2B and 2C), supporting the concept that exosomal signaling augments the invasive phenotype

Figure 4. Pretreatment of EVs with antibody against FN blocks invasion of nonsenescent lung fibroblasts. EVs (500 ng) were incubated with FN antibody of varying dilution for 30 min and seeded in Matrigel as described in METHODS. (A) Nonsenescent lung fibroblasts were allowed to invade for 16 hours through Matrigel containing EVs pretreated with FN antibody. Scale bars: 100 μ m. (B) The bar graph represents the number of invasive fibroblasts per high-power field ($\times 400$). Data are mean $\pm$ SEM; $n = 3$ technical replicates; five random fields were counted for each replicate; $*P < 0.0001$ (vs. 1:10,000 dilution). (C) The experiment was repeated using mouse IgG1 as the isotype control for the FN antibody. Scale bars: 50 μ m. (D) The bar graph represents the number of invasive fibroblasts per high-power field ($\times 400$). Data are mean $\pm$ SEM; $n = 3$ technical replicates; five random fields were counted for each replicate; $*P < 0.05$ (EV+FN-Ab vs. EV+IgG1); $**P < 0.01$ (EV vs. EV+FN-Ab). (E) Nonsenescent lung fibroblast-derived EVs were fluorescently labeled with the nucleic acid-specific dye acridine orange (Exo-Red). Labeled EVs were incubated with mouse anti-human FN antibody or mouse IgG1 *in vitro* for 30 minutes and added to the nonsenescent lung fibroblasts in chambered slides. Images were obtained 30 minutes after the EVs were added to the fibroblasts. Scale bars: 50 μ m. Ab = antibody.

of lung fibroblasts. No significant difference in invasive capacity was noted between EVs isolated from senescent versus nonsenescent fibroblasts (Figure E4).

Next, we determined whether direct interaction of soluble EVs with fibroblasts is sufficient to augment fibroblast invasion or whether this is dependent on its interaction with the ECM. Fibroblasts were incubated with 500 ng EVs for 1 hour in culture, and the EVs were then removed by centrifugation and plated on top of Matrigel invasion chambers. Interestingly, fibroblast invasion through Matrigel was increased to an extent similar to that observed after addition of EVs to the Matrigel (Figures 2D and 2E), suggesting that exosomal signaling of fibroblast invasion can be mediated by a direct interaction of EVs with cells.

FN Is Expressed on the Surface of Fibroblast-derived EVs

To determine whether FN is present on the surface of EVs, we analyzed purified EVs by flow cytometry. EVs were dual immunostained with PE-labeled human FN antibody (R&D Systems) and eFluor 450-labeled EV marker CD63 antibody (eBioscience), and subjected to flow-cytometric analysis. The vast majority of EVs isolated from nonsenescent fibroblasts showed surface expression of FN (Figure 3A). Additionally, dual staining with FN-PE and eFluor 450-CD63, analyzed by ImageStream, confirmed surface coexpression of FN and CD63 (Figure 3B).

FN on the Surface of EVs Mediates Fibroblast Invasion

FN has been shown to serve as a chemoattractant for lung fibroblasts (18); however, it is not known whether EV-associated FN promotes fibroblast invasion. To confirm that surface FN was responsible for EV-mediated fibroblast invasion, fibroblast-derived EVs were preincubated with varying dilutions (1:100, 1:1,000, 1:5,000, and 1:10,000) of antibody against human FN (Millipore-SIGMA; IST-4). Antibody-treated EVs (500 ng) in growth medium were mixed with Matrigel in a 1:1 ratio, allowed to solidify at 37°C, and subjected to fibroblast invasion. We demonstrated a dose-dependent inhibition of fibroblast invasion. Mouse IgG1, an isotype of the FN antibody, was used as control (Figures 4A–4D). These findings clearly

demonstrated that EV surface expression of FN is crucial for lung fibroblast migration and invasion.

To determine whether surface expression of FN facilitated EV interaction with the lung fibroblasts, EVs were first fluorescently labeled with the nucleic acid-specific dye acridine orange (Exo-Red). Labeled EVs were then incubated *in vitro* with either mouse IgG1 or antibody against the mouse FN. EVs were then added to the nonsenescent lung fibroblasts in culture and observed for EV cargo delivery. The results indicated a significantly inefficient transfer of fluorescently labeled nucleic acid cargo from EVs pretreated with FN antibody when compared with IgG1-treated and -untreated controls (Figure 4E), suggesting that FN is critical for mediating EV interaction with recipient lung fibroblasts.

Integrin $\alpha 5\beta 1$ is a known ligand of FN (19). To determine whether EV-mediated fibroblast invasion involves FN- $\alpha 5\beta 1$ interaction, we performed fibroblast invasion assays in the presence and absence of a mouse monoclonal antibody against human $\alpha 5\beta 1$ integrin (Millipore-SIGMA), with mouse IgG1 used as control. This resulted in a complete inhibition of fibroblast invasion (Figures 5A and 5B).

FN-EV Interaction with Fibroblasts Activates Invasion-related Signaling

Cellular migration/invasion requires integrin-mediated signaling via canonical protein kinases involving focal adhesion kinase (FAK) and Src family kinases (20, 21). To determine whether these kinase pathways were activated by exosomal signaling, fibroblasts were incubated with EVs (500 ng, as described above) and subjected to Western blot analysis for expression of phosphorylated (activated) FAK and phosphorylated (activated) Src kinase. Activation of FAK and Src was observed in fibroblasts exposed to EVs (Figures 6A–6C), suggesting that a direct interaction between fibroblasts and EVs via FN results in the activation of invasion-related signaling pathways.

To further confirm that the EV-mediated activation of invasion-associated signaling pathways in the lung fibroblasts involves FAK and Src family kinases, we performed an invasion assay in the presence of AG1879, a potent inhibitor of the Src family of kinases. The results indicated a

significant inhibition of fibroblast invasion compared with control (EV only). To determine whether the effect of FN-blocking antibodies on fibroblast invasion was due to steric hindrance and not to specific antagonism of FN binding to the fibroblast surface, we also performed an invasion assay in the presence of an antibody against CD63, an EV surface marker. No inhibition of fibroblast invasion was observed with CD63 antibody, suggesting a specificity of the EV-fibroblast interaction through FN (Figures 7A and 7B).

Discussion

There is growing recognition of the role of EVs in cell-cell communication (22–24). Alterations in quantitative and qualitative EV signaling may contribute to the pathogenesis of diseases, including those

Figure 5. Pretreatment of EVs with antibody against integrin $\alpha 5\beta 1$ blocks invasion of nonsenescent lung fibroblasts. (A) EVs (500 ng) were incubated with integrin $\alpha 5\beta 1$ antibody of 1:100 dilution for 30 minutes and seeded in Matrigel as described in METHODS. Nonsenescent lung fibroblasts were allowed to invade for 16 hours through Matrigel containing EVs in the absence and presence of integrin $\alpha 5\beta 1$ antibody. Scale bars: 50 μ m. (B) The bar graph represents number of invasive fibroblasts per high-power field ($\times 400$). Data are mean $\pm$ SEM; $n = 3$ technical replicates; five random fields were counted for each replicate; * $P < 0.0001$ (vs. EV).

Figure 6. Activation of focal adhesion kinase (FAK)-Src signaling in the lung fibroblasts. (A) Nonsenescent lung fibroblasts were incubated with nonsenescent-derived EVs (500 ng) for 1 hour and subjected to Western blotting analysis for upregulation of invasion-associated genes. Readers may view the uncut gels for A in the data supplement. (B and C) Densitometry was performed to quantitate the ratio of p-FAK (B) and p-Src (C) to β -actin (loading control) and plotted. The bar graphs represent mean $\pm$ SEM, $n = 3$; * $P < 0.05$ compared with control. All of the experiments were performed in triplicate at each time and repeated at least twice.

associated with aging. In this study, we have demonstrated that EVs from senescent fibroblasts bind to the ECM, and this sequestration is severalfold higher in senescent ECM than in nonsenescent ECM. EVs from both senescent and nonsenescent fibroblasts induce an invasive phenotype of recipient fibroblasts. This effect is mediated through FN bound on the EV surface, which binds to integrin $\alpha 5 \beta 1$ and activates invasion signaling involving FAK and Src kinases. This is the first report, to our knowledge, of a paracrine/autocrine role of EV-associated FN in lung fibroblast invasion, a critical event in the pathogenesis of fibrotic diseases (18).

FN alone has been shown to act as a chemoattractant for fibroblast invasion (25).

It is possible that binding of FN on the surface of EVs leads to augmented fibroblast invasion as compared with equimolar concentrations of free FN. However, this would be a challenging concept to test due to the difficulty of precisely measuring the amount of FN carried on EVs. In support of more efficient signaling by EV surface-associated FN, it is known that cryptic sites in soluble (globular) FN that are inaccessible to bind integrins can do so efficiently when FN undergoes fibrillogenesis on biological surfaces (26). Further studies are required to clarify the role of FN signaling when it is present on EV surfaces.

EVs participate in intercellular communication and are integral

components of the ECM (24, 27–31). They have been shown to mediate matrix remodeling, tissue injury repair, and tumor invasion through promotion of cell migration as well as ECM degradation (3, 4, 32–34). EV signaling is mediated by a number of mechanisms: 1) EV membranes fuse with the plasma membrane of target cells and release its contents into the cytoplasm to activate downstream signaling events (35); 2) EVs release proteinases into the surrounding ECM to generate bioactive ECM fragments (23); and 3) proteins expressed on the surface of EVs function as ligands to activate receptors on target cells (3, 36–39). In this study, we demonstrated that the effect of EVs on target cells is mediated via interaction of EVs with target cells through FN-integrin $\alpha 5 \beta 1$ interaction. Our findings are similar to previous observations that FN on the surface of myeloma-derived exosomes can bind to target cells and promote endothelial cell invasion (4). Our findings show that activation of FAK/Src signaling in lung fibroblasts exposed to EVs may be due to engagement of integrin $\alpha 5 \beta 1$ on the surface of recipient cells. Alternatively, this surface interaction involving FN facilitates delivery of EV cargo to the recipient fibroblasts. Typically, EV cargo uptake by the recipient cell involves protein interactions, which facilitates subsequent endocytosis mediated by clathrin (40, 41), caveolin (42, 43), and lipid rafts (44). Our findings support a role for FN as a necessary component of EVs in mediating cell–cell communication.

Both fibroblast invasion and cellular senescence have been linked to the pathogenesis of fibrotic lung disease (7, 8). Studies have shown that senescent cells secrete a significantly greater amount of EVs than nonsenescent cells (45–47). Consistent with these studies, we observed that senescent fibroblasts produced greater amounts of EVs than their nonsenescent counterparts. We found no significant difference in fibroblast invasion when we compared EVs from senescent and nonsenescent fibroblasts. Interestingly, in senescent lung fibroblasts, >90% of the EVs were bound to the ECM, compared with <20% in nonsenescent cells. It is unclear whether the higher number of EVs in the senescent matrix is the result of a higher number of EVs produced by senescent cells, or whether increased “adhesiveness” of the senescent matrix may also contribute to a greater “sequestration”

Figure 7. Inhibition of FAK-Src signaling in lung fibroblasts blocks invasion. (A) Nonsenescent lung fibroblasts were allowed to invade for 16 hours through Matrigel containing EVs in the presence of AG1879, a selective inhibitor of FAK-Src signaling. EV-mediated fibroblast invasion was also performed in the presence of CD63 (EV surface marker) antibody to determine the specificity of FN-blocking antibodies shown in Figure 4. Scale bars: 50 μ m. (B) The bar graph represents number of invasive fibroblasts per high-power field ($\times 400$). Data are mean $\pm$ SEM; $n = 3$ technical replicates; five random fields were counted for each replicate; * $P < 0.0001$ (vs. EV only).

within the matrix. Interestingly, a lower sedimentation rate was observed in EVs derived from senescent fibroblasts in comparison with nonsenescent fibroblasts,

which may have resulted in an underestimation of the number of EVs released by senescent cells. Lipids are essential components of EV membranes

(48). Variable lipid composition as well as adhesiveness and/or aggregation may contribute to differences in sedimentation rates. We observed that replication-induced senescence led to significantly greater numbers of released EVs in comparison with TGF- β 1 stimulation of the same cells; furthermore, control adult fibroblasts produced severalfold lower numbers of EVs at baseline compared with fetal lung fibroblasts, indicating important biological differences between these cell types. Importantly, replication-induced senescence, TGF- β 1 stimulation, and IPF resulted in greater release of EVs relative to their respective controls. Our studies suggest that EVs anchored in the senescent ECM may contribute to the profibrotic microenvironment that characterizes chronic fibrotic diseases.

This is the first study, to our knowledge, to demonstrate a role for EVs in fibroblast invasion. Our report supports autocrine and paracrine roles for EVs in fibroblast migration/invasion relevant to the pathogenesis of fibrotic disorders. Targeting these fibroblast-EV interactions may prove to be an effective therapeutic strategy for fibrosis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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