Fibronectin on the Surface of Myeloma Cell-derived Exosomes Mediates Exosome-Cell Interactions*

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Exosomes regulate cell behavior by binding to and delivering their cargo to target cells; however, the mechanisms mediating exosome-cell interactions are poorly understood. Heparan sulfates on target cell surfaces can act as receptors for exosome uptake, but the ligand for heparan sulfate on exosomes has not been identified. Using exosomes isolated from myeloma cell lines and from myeloma patients, we identify exosomal fibronectin as a key heparan sulfate-binding ligand and mediator of exosome-cell interactions. We discovered that heparan sulfate plays a dual role in exosome-cell interaction; heparan sulfate on exosomes captures fibronectin, and on target cells it acts as a receptor for fibronectin. Removal of heparan sulfate from the exosome surface releases fibronectin and dramatically inhibits exosome-target cell interaction. Antibody specific for the Hep-II heparin-binding domain of fibronectin blocks exosome interaction with tumor cells or with marrow stromal cells. Regarding exosome function, fibronectin-mediated binding of exosomes to myeloma cells activated p38 and pERK signaling and expression of downstream target genes DKK1 and MMP-9, two molecules that promote myeloma progression. Antibody against fibronectin inhibited the ability of myeloma-derived exosomes to stimulate endothelial cell invasion. Heparin or heparin mimetics including Roneparstat, a modified heparin in phase I trials in myeloma patients, significantly inhibited exosome-cell interactions. These studies provide the first evidence that fibronectin binding to heparan sulfate mediates exosome-cell interactions, revealing a fundamental mechanism important for exosome-mediated cross-talk within tumor microenvironments. Moreover, these results imply that therapeutic disruption of fibronectin-heparan sulfate interactions will negatively impact myeloma tumor growth and progression.

Multiple myeloma remains incurable, despite recent treatment advances (1). There is mounting evidence that targeting intercellular communication between malignant plasma cells or between malignant cells and host cells may enhance therapeutic outcome. Extracellular vesicles, because they can directly interact with target cells, have emerged as important mediators of intercellular communication. Exosomes are a type of extracellular vesicle derived from endosomes and released by cells into the extracellular space. Exosomes are generally characterized by their size (ranging from ~30 to 150 nm) and expression of certain surface markers. However, at present there are no markers specific for exosomes (2). Several studies have shown that these exosome-like particles secreted by tumor cells can affect survival, apoptosis, invasion, angiogenesis, resistance to therapy, and premetastatic niche formation (3–6). Exosomes influence these processes by mediating signaling at the cell surface and by facilitating the intercellular transfer of tumor-derived proteins and nucleic acids. Recent studies have demonstrated critical roles for myeloma-derived exosomes in mediating intercellular communication and promoting myeloma progression (7–9). For example, myeloma cell-derived exosomes can promote the formation and function of osteoclasts and thereby stimulate osteolysis (7). Another study showed that exosomal microRNA 135b released by hypoxic multiple myeloma cells enhances angiogenesis by targeting factor inhibiting hypoxia-inducible factor 1 (8). In addition, we recently discovered that exosomes secreted by aggressive myeloma cells can induce tumor cell spreading and endothelial cell invasion (9).

Heparan sulfate proteoglycans are glycoproteins that contain a core protein with one or more covalently attached negatively charged heparan sulfate glycosaminoglycan chains. Heparan sulfate chains can bind and regulate the function of a wide variety of adhesion molecules, cytokines, growth factors, chemokines, morphogens, various types of viral particles, lipoproteins, etc. (10). By interacting with various cell adhesion receptors and extracellular matrix proteins such as fibronectin and collagens, heparan sulfate chains promote cell-cell and cell-ECM interactions (10). Although the functional effects of exosomes rely on their interaction with, and subsequent delivery of their cargo to target cells, the mechanisms mediating exosome-cell interactions remain unclear. Studies have shown that heparin can block interactions between extracellular vesicles and cells and that heparan sulfate proteoglycans on glioblastoma cells facilitates extracellular vesicle uptake (11–14). This uptake was

blocked by the addition of exogenous heparin (a highly sulfated glycosaminoglycan mimetic of heparan sulfate), which can compete with cell surface heparan sulfate to bind exosomes. However, the receptor on the exosome surface that binds to heparan sulfate on the target cell has not been identified.

In the present study, we discovered that fibronectin on the exosome surface mediates the interaction with heparan sulfate chains on target cells. Fibronectin is a high affinity ligand for heparin/heparan sulfate and contains a strong heparin/heparan sulfate binding site, designated Hep-II, located within the C-terminal repeat units 12–14 of fibronectin (15). Several studies have shown that binding of cell surface heparan sulfate proteoglycans such as syndecans to the heparin-binding domain on fibronectin or to a synthetic peptide corresponding to this domain induces the formation of stress fiber, focal adhesion kinase, and cell spreading (16–18). We demonstrate that myeloma-derived exosomes have fibronectin bound on their surface and that, via its Hep-II domain, fibronectin facilitates exosome interaction with heparan sulfate chains present on the surface of target cells. Additionally, we show that fibronectin is bound to exosome surface via interacting with heparan sulfate chains on the exosome surface. These findings provide the first evidence that fibronectin present on the surface of tumor-derived exosomes facilitates exosome-target cell interaction and reveal a novel mechanism whereby the heparan sulfate proteoglycans present on exosomes and on target cells can influence exosome-mediated intercellular communication.

Experimental Procedures

Cell Lines—RPMI-8226 and CAG human myeloma cells were cultured as described previously (19). Aggressive (heparanase-high) and less aggressive (heparanase-low) control CAG cells were selected as described previously (20). For preparing cells to secrete fluorescent exosomes, CAG cells were infected with lentivirus particles packaged with pCT-CMV vector encoding CD63 tetraspanin-red fluorescent protein (RFP)[3] fusion protein (System Biosciences). Infected cells were selected using puromycin. Human bone marrow stromal cell line HS-5 and human umbilical vein endothelial cells were maintained as described previously (21).

Exosome Isolation from Cell Culture Supernatants and Myeloma Patient Serum—Human myeloma cells growing in culture were washed twice with PBS and grown in serum-free medium for 24–42 h. As previously described, exosome-like particles secreted into the medium by CAG myeloma cells were isolated by differential centrifugation, characterized by surface marker expression and size, and found to be consistent with the characteristics of exosomes (9). Particle size was measured by nanoparticle tracking analysis using a NanoSight 300 (Malvern Instruments Ltd.). For mass spectrometry and flow cytometry analyses, 800 μg of exosomes obtained by ultracentrifugation were layered on the top of a 40% iodixanol cushion (Sigma) and centrifuged for 16 h at 28,000 rpm. The remaining exosome fraction excluded by the cushion was pelleted by ultracentrifugation at 100,000 $\times$ g for 70 min and used for analysis.

Exosomes were quantified by nanoparticle tracking analysis or by measuring the protein using a BCA protein assay kit (Pierce).

Serum samples were obtained from treatment naïve multiple myeloma patients enrolled in the Molecular and Genetic Epidemiology (iMAGE) study of myeloma who met the revised and updated International Multiple Myeloma Working Group classification criteria for myeloma (22). Approvals from the appropriate Institutional Review Boards were obtained prior to study initiation. Exosomes were isolated from serum using an ExoQuick isolation kit (System Biosciences). Briefly, to 100 μl of serum, 30 μl of ExoQuick solution was added and incubated at 4 °C for 1 h and then centrifuged at 1500 $\times$ g for 30 min. The pellet was resuspended in PBS, and the exosomes were further purified using anit-CD63 conjugated to magnetic beads (System Biosciences), according to the manufacturer's instructions. Particle size and number was assessed using NanoSight 300. The capture settings and analysis settings were performed manually according to the manufacturer's instructions. For some experiments, exosomes were fluorescently labeled using PKH67 (green) or PKH26 (red) (Sigma), according to the manufacturer's recommendation, followed by extensive washing to remove residual lipid dye.

Flow Cytometry Analysis of Exosomes Bound to Beads—Flow cytometry analysis to identify molecules on the surface of exosomes was performed after attaching exosomes to either anti-CD63-bound beads or heparin-agarose beads (MP Biomedicals Inc.). 100 μg of purified exosomes were mixed with the anti-CD63 beads or heparin agarose beads and incubated on a rotating rack at 4 °C overnight. Exosomes bound to beads were suspended in 200 μl of 1% BSA in PBS and stained with antibodies against fibronectin or syndecan-1 prior to analysis with a Becton Dickinson FACSCalibur flow cytometer located in the UAB Comprehensive Flow Cytometry Core. Fibronectin was stained using a mouse monoclonal anti-human fibronectin-PE-conjugated antibody (R&D Systems). Mouse isotype matched (IgG1) PE (Thermo Fisher) was used as the control. For detection of syndecan-1, exosomes bound to anti-CD63 beads were treated with bacterial heparitinase (Seikagaku) for 2 h at 37 °C followed by extensive washing. This enzyme treatment, by releasing heparan sulfate and any bound ligands (*e.g.* fibronectin), exposes the core protein epitope to the antibody. Syndecan-1 was detected using an affinity-purified polyclonal goat anti-syndecan-1 IgG (R&D Systems) and PE-conjugated secondary antibody. Normal goat IgG was used for the control (Santa Cruz).

Exosome Protein Analysis by MS/MS—Exosomes excluded by an iodixanol cushion were solubilized in $1\times$ LDS sample buffer (NuPAGE; Life Technologies) followed by membrane disruption for 10 min in an ultrasonic bath (Thermo Fisher) and heat denaturation as per manufacturer's instructions for the LDS buffer. Protein extracts were then quantified using the BCA protein assay kit (Pierce, Life Technologies). An aliquot containing 20 μg of protein was reduced, denatured, and loaded onto a 10% Bis-Tris gel (NuPAGE reagents; Life Technologies) and separated as a short stack run ($\sim$1 cm). The gel was stained with a colloidal blue staining kit (NuPAGE, Life Technologies), destained, and visualized. The upper gel section containing protein for each sample was cut out and digested using Trypsin

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[3] The abbreviations used are: RFP, red fluorescent protein; MMP, matrix metalloproteinase; PE, phycoerythrin.

Gold (Promega), followed by peptide extraction as per the manufacturer's instructions, and the volumes were reduced using a Savant SpinVac Concentrator (Thermo Fisher). One microgram of peptide extract (diluted to ~1 μg/10 μl in 0.1% formic acid) was loaded onto a 100 μm $\times$ 13-cm capillary column, packed in-house with C18 Monitor 100 A-spherical silica beads, and eluted over a 90-min gradient (0–30% acetonitrile in 0.1% TFA). Liquid chromatography mass spectrometric analysis was performed in duplicate using an LTQ Velos Pro Orbitrap (Thermo Fisher), and data were analyzed within the University of Alabama at Birmingham Comprehensive Cancer Center Mass Spectrometry/Proteomics Shared Facility as previously described (23).

Analysis of Exosome-Cell Interactions—Subconfluent RPMI-8226 myeloma cells or HS-5 bone marrow stromal cells were incubated with CD63-RFP or PKH-labeled myeloma-derived exosomes (100 μg/ml) for 2 h. The cells were washed in PBS and fixed in 3% paraformaldehyde for 15 min on ice. Myeloma cells grow in suspension and therefore were cytospun on to slides for analysis. All samples were analyzed using a Nikon A1 confocal microscope. Fluorescent exosomes bound to cells were quantified by flow cytometry using a FACSCalibur instrument. For experiments designed to block the Hep-II domain of fibronectin on exosomes, the exosomes were preincubated with monoclonal antibody (A32) (25 μg of antibody/100 μg of exosome) that binds specifically to the Hep-II heparin-binding domain of fibronectin (Thermo Fisher). For experiments designed to block heparan sulfate on the surface of target cells, the cells were incubated with a 40-kDa fragment of human fibronectin (purified from chymotryptic digest of human plasma fibronectin) that contains the Hep-II binding domain (50 μg/ml) (Millipore). After washing to remove unbound fibronectin fragments, exosome-target cell interaction was analyzed by confocal microscopy or flow cytometry. For experiments in which heparan sulfate was removed from cell surface, cells were treated with 5 millunits/ml of bacterial heparitinase for 2 h at 37 °C, followed by extensive washing. For removing heparan sulfate from exosomes, 100 μg of exosomes were treated with 1.5 millunits/ml heparitinase for 3 h at 37 °C; new enzyme was added, and exosomes were incubated overnight. Exosomes were then pelleted by ultracentrifugation to remove enzyme. In some blocking experiments, exogenous heparin (10 μg/ml), Roneparstat (10 μg/ml), or a synthetic heparin dodecasaccharide (100 μg/ml) was added simultaneously with 100 μg of exosomes/ml. Roneparstat (previously known as SST0001) is a proprietary drug of Sigma-Tau Research Switzerland that inhibits growth of myeloma tumors in animal models (24) and is currently in phase I trials in advanced multiple myeloma patients (ClinicalTrials.gov identifier NCT01764880). The heparin dodecasaccharide is a fully sulfated molecule prepared by chemoenzymatic synthesis (25).

Fibronectin ELISA—Fibronectin levels were quantified using a commercially available human fibronectin ELISA kit (Millipore), according to the manufacturer's protocol. The assay is in the format of competitive inhibition ELISA. For some experiments, exosomes were treated without or with 1.5 millunits/ml heparitinase for 3 h at 37 °C followed by the addition of fresh enzyme and incubation overnight. Exosomes were then washed and pelleted by ultracentrifugation or ExoQuick exosome precipitation solution. Exosome protein content of the various samples was determined by BCA protein assay, and an equal amount of exosome protein (8 μg) from each sample was used for the ELISA.

Western Blot—100 μg of exosomes, based on BCA protein assay, were lysed in RIPA buffer containing protease inhibitors for 10 min on ice, and proteins were separated by electrophoresis in a 4–12% SDS-PAGE. The proteins were electroblotted to nitrocellulose membrane and probed with primary antibodies that recognize fibronectin (R&D Systems), clathrin (Abcam), or flotillin (Abcam). In some experiments, the exosomes were treated with bacterial heparitinase and probed for syndecan-1 (R&D Systems). Primary antibodies were detected using HRP-conjugated secondary antibodies, and bands were visualized by chemiluminescence. In some experiments, RPMI-8226 cells were treated with or without heparitinase for 2 h at 37 °C and then exposed to 100 μg/ml of either control or aggressive exosomes for 20 min. Cells were lysed and probed for phosphorylated p38 or total p38 (Cell Signaling).

Real Time PCR—RNA was isolated from CAG human myeloma cells incubated with or without exosomes, and real time PCR was conducted for MMP-1 and DKK1 using SYBER Green supermix (Bio-Rad). mRNA expression was determined relative to actin.

Human Phosphokinase Array—A membrane-based antibody array (R&D Systems) that determines the relative levels of 43 different human phosphorylated protein kinases was utilized according to the manufacturer's instructions. Briefly, equal amounts of cell lysates of human RPMI-8226 cells treated without or with 100 μg of exosomes were incubated overnight with the phosphokinase array membrane. The array was washed to remove unbound proteins followed by incubation with a mixture of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied to visualize the signal produced at each capture spot corresponding to the amount of phosphorylated protein bound.

Endothelial Cell Invasion—The effect of exosomes on the invasion of human umbilical vein endothelial cells was assessed using Biocoat Matrigel invasion chambers (BD Biosciences), as described (9). To study the relevance of fibronectin-heparan sulfate interactions for the functional activity of exosomes, exosomes were preincubated without or with the anti-fibronectin antibody A32 (25 μg of antibody/100 μg of exosome protein) before adding to the endothelial cells.

Results

Fibronectin Is Present on the Surface of Myeloma-derived Exosomes and Enhances Exosome-Cell Interactions—Expression of the heparan sulfate degrading enzyme heparanase in CAG human myeloma cells reprograms them toward a highly aggressive phenotype *in vivo* endowing tumors formed by these heparanase-high cells with increased metastasis, angiogenesis, and osteolysis compared with control, heparanase-low expressing cells (26–29). In analyzing exosomes from these aggressive and control cells, we found those secreted by the aggressive cells had higher levels of syndecan-1 (CD138), VEGF, and hepatocyte growth factor and were able to promote tumor cell
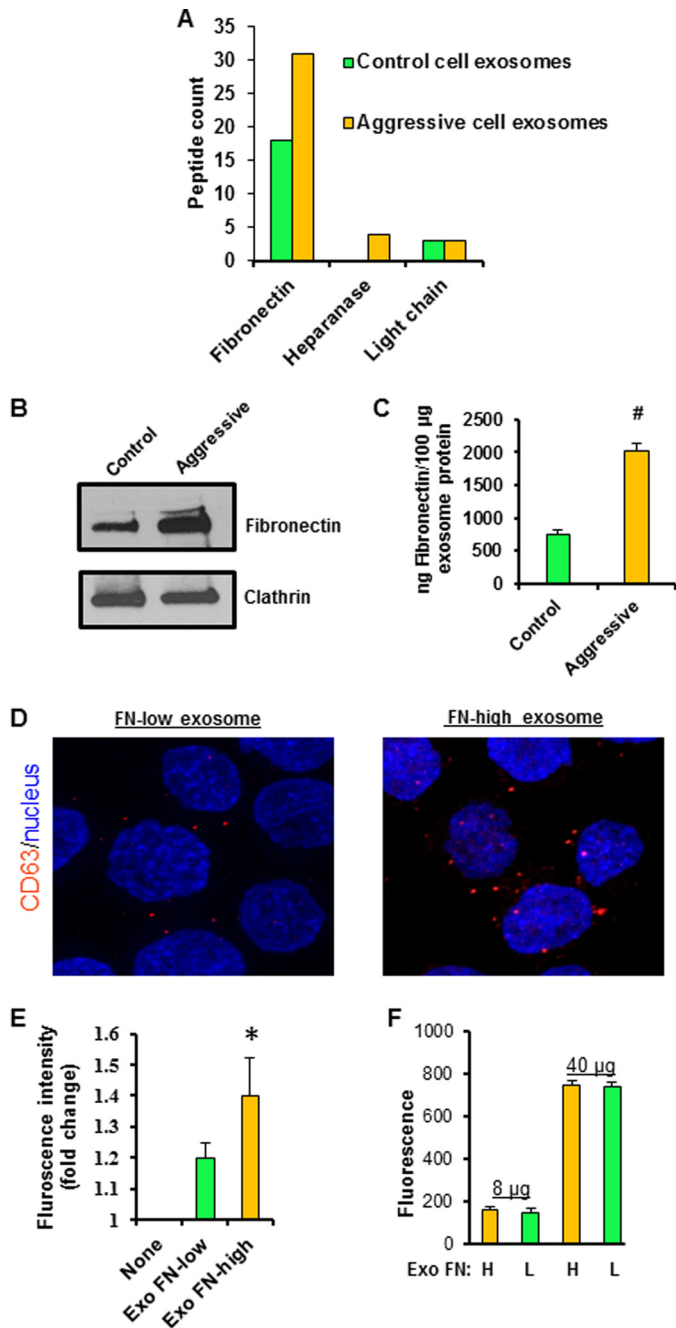
FIGURE 1. **Fibronectin is present on myeloma-derived exosomes, and a high level of fibronectin correlates with enhanced exosome interaction with target cells.** *A*, result of a single mass spectrometry analysis of selected exosomal proteins from human CAG control (heparanase-low) or aggressive (heparanase-high) myeloma cells. Fibronectin was the protein most enhanced in abundance in exosomes from aggressive cells compared with control. *B* and *C*, the amounts of fibronectin associated with exosomes secreted by control or aggressive CAG myeloma cells were quantified by Western blot (*B*) and ELISA (*C*). The results are means ± S.D. of three independent experiments. #, $p < 0.05$ compared with control. Clathrin, a marker for cell-derived vesicles, serves as the loading control for the Western blot. *D*, to generate cells that secrete CD63-RFP exosomes, CAG control or aggressive cells were transfected with a lentiviral vector coding for a CD63-RFP chimeric protein. Exosomes (10 μg/ml) secreted by these cells were incubated with human RPMI-8226 myeloma cells. After 2 h, cells were washed, fixed with 3.7% formaldehyde, cytospun, and imaged by confocal microscopy. *Red*, RFP exosomes; *blue*, nucleus. *E*, quantitative data from an experiment similar to that shown in *D* were obtained by subjecting the cells with bound exosomes to flow cytometry. The data are presented as fold increase of median fluorescence intensity of cells incubated with exosomes as compared with cells not incubated with exosomes and are mean ± S.D. from three independent

spreading and endothelial cell migration much better than exosomes from control cells, which failed to substantially impact cell behavior (9). To look for other exosomal proteins that regulate exosome function, we compared the protein content of exosomes secreted by the aggressive *versus* control cells using mass spectrometry. As expected, the level of heparanase detected was much higher in exosomes secreted by the aggressive CAG cells, whereas the level of immunoglobulin light chain was identical in the exosomes secreted by control and aggressive CAG cells (Fig. 1*A*). Interestingly, fibronectin was significantly enhanced in abundance in exosomes from aggressive cells compared with control cells. The presence of fibronectin in exosomes was confirmed by Western blotting and ELISA (Fig. 1, *B* and *C*).

Interaction of exosomes with target cells is a critical step in the process of exosome-mediated intercellular communication. Because fibronectin is known to play an important role in adhesion via interactions with integrins, collagens, fibrin, and heparan sulfate proteoglycans, we hypothesized that it played a role in exosome-cell interactions. First, to determine whether the level of fibronectin on exosomes correlated with their ability to interact with target cells, we transfected the aggressive and control CAG cells with a construct coding for a chimeric protein composed of CD63 and red fluorescent protein (CD63-RFP). CD63 is a tetraspanin protein that is abundant on the exosome membrane. Thus cells transfected with the CD63-RFP secrete exosomes that can easily be tracked by fluorescence microscopy. Interestingly, by both confocal microscopy and flow cytometry, we found that the exosomes secreted by the aggressive cells and containing high levels of fibronectin interacted much better with target cells than did the exosomes having low levels of fibronectin (Fig. 1, *D* and *E*). As shown in Fig. 1*F*, the RFP levels in the exosomes secreted by aggressive and control cells were equivalent, thereby validating their use for quantitative studies. Having established that exosomes having high levels of fibronectin readily interact with target myeloma cells, we utilized these exosomes in all the subsequent *in vitro* studies to explore the role of fibronectin in exosome-cell interactions.

In initial studies we assessed whether fibronectin was localized to the exosome surface. For this, exosomes were isolated by centrifugation over an iodixanol cushion and bound to either anti-CD63-containing magnetic beads (Fig. 2*A*, *left panel*) or heparin-agarose beads (Fig. 2*A*, *right panel*), exposed to anti-fibronectin antibody, and subjected to flow cytometry. The results demonstrated that fibronectin is abundant on the exosome surface. Because fibronectin can bind to heparan sulfate and because these exosomes from aggressive CAG cells have a high level of the heparan sulfate-bearing proteoglycan syndecan-1 on their surface (9), we speculated that fibronectin may be bound to the exosome surface through its interaction with heparan sulfate chains of syndecan-1. To test this, exosomes

experiments. *, $p < 0.05$. *F*, to ensure that the results in *D* and *E* above were not due to a higher per exosome concentration of CD63-RFP in the exosomes secreted by aggressive cells, the RFP intensity of an equal amount (8 or 40 μg) of exosomes (*exo*) with low (*L*) and high (*H*) levels of fibronectin was measured. The fluorescence intensity (excitation, 565 nm; emission, 610 nm) of exosomes was equivalent. *FN*, fibronectin.
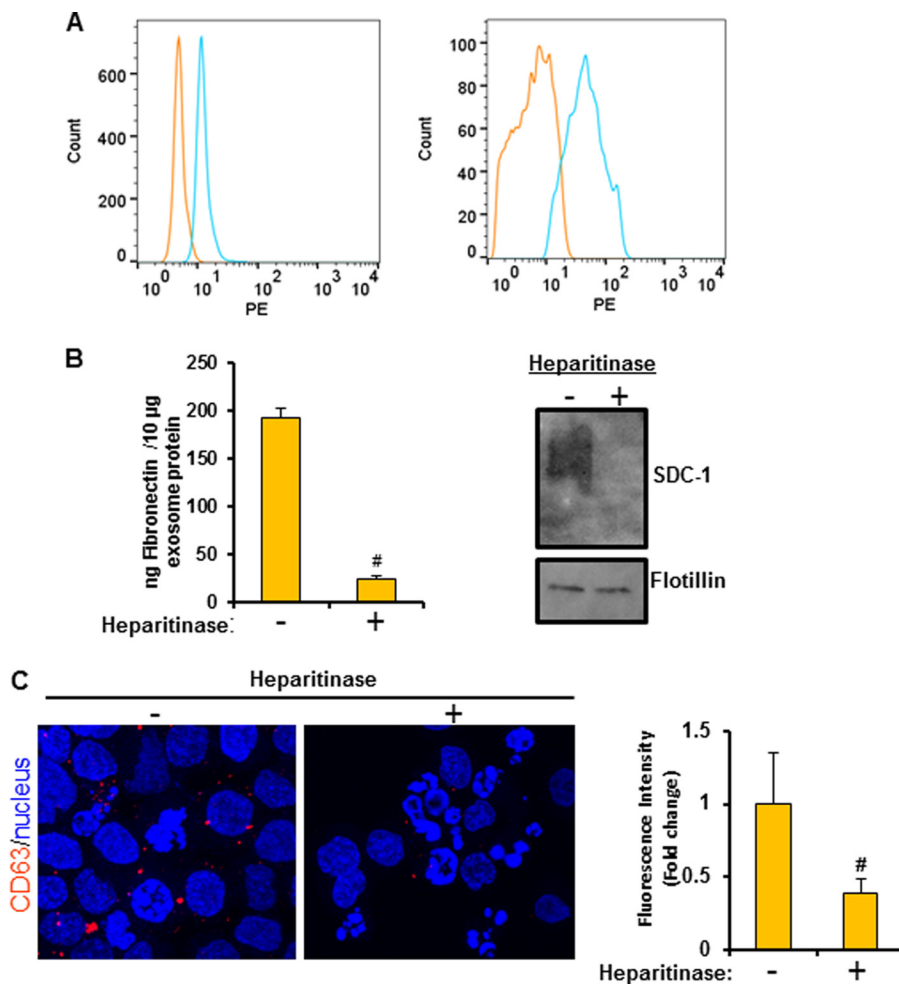
FIGURE 2. **Fibronectin is present on the exosome surface and its removal inhibits exosome interaction with target cells.** *A*, fibronectin is present on the surface of exosomes. Exosomes from aggressive CAG cells, purified by ultracentrifugation and excluded by an iodixanol cushion, were captured either using anti-CD63 magnetic beads (*left panel*) or using heparin-agarose beads (*right panel*) and subjected to flow cytometry analysis using a mouse monoclonal PE-conjugated anti-fibronectin antibody (*blue histogram*). PE-conjugated isotype-matched antibody was used as a control (*orange histogram*). *B*, removal of heparan sulfate from the exosome surface removes most of the fibronectin associated with the exosome. *Left panel*, exosomes were either not treated or treated with bacterial heparitinase (1.5 millunits/ml heparitinase for 3 h at 37 °C followed by addition of fresh enzyme and incubation overnight), a heparan sulfate degrading enzyme, and fibronectin levels were quantified by ELISA. The data are expressed as means $\pm$ S.D. #, $p < 0.05$ compared with exosomes not treated with heparitinase. *Right panel*, Western blot for syndecan-1 on exosomes after heparitinase digestion. Flotillin, a marker for cell-derived vesicles, serves as the loading control. *C*, depletion of heparan sulfate from exosomes decreases exosome-target cell interaction. CD63-RFP exosomes secreted by CAG cells were either untreated or treated with heparitinase and washed, and their interactions with RPMI-8226 cells were analyzed by confocal microscopy. *Right panel*, quantitative data from a similar experiment analyzed by flow cytometry. The results are expressed as fold change of the median fluorescence intensity. #, $p < 0.05$ compared with no heparitinase treatment.

were exposed to bacterial heparitinase, an enzyme that extensively degrades the heparan sulfate chains of syndecan-1 on the surface of these exosomes (Fig. 2*B*, *right panel*). Following the removal of heparan sulfate from the exosome surface by heparitinase, the fibronectin remaining associated with the exosome was greatly reduced (Fig. 2*B*, *left panel*). To determine whether exosomes depleted of fibronectin from their surface affected exosome-target cell interaction, CD63-RFP exosomes were treated with heparitinase, pelleted to remove the enzyme, and then incubated with RPMI-8226 myeloma cells. Fluorescence microscopy and flow cytometry revealed that removal of fibronectin from the exosome surface dramatically reduced their binding to target cells compared with exosomes having fibronectin on their surface (Fig. 2*C*). Together these results indicate that fibronectin is present on the surface of exosomes, it is retained there via interaction with heparan sulfate chains,

and its removal inhibits interaction of exosomes with target cells.

Fibronectin on Exosomes Binds to Heparan Sulfate Chains on Target Cells—It was previously demonstrated that exosomes secreted by human glioblastoma cells interact with heparan sulfate chains present on target cells (13). Based on this observation, we speculated that fibronectin, in addition to interacting with heparan sulfate on the exosome surface, was also interacting with heparan sulfate on the surface of target cells, thereby serving as an adhesive "linker" between exosome and target cells. This was first examined by incubating exosomes with polarized or nonpolarized CAG myeloma cells; polarized cells have heparan sulfate localized to the uropod of cells, and nonpolarized cells have heparan sulfate distributed over the cell surface (19). Confocal microscopy revealed that the exosomes bound to these cells in a pattern that mimicked this known
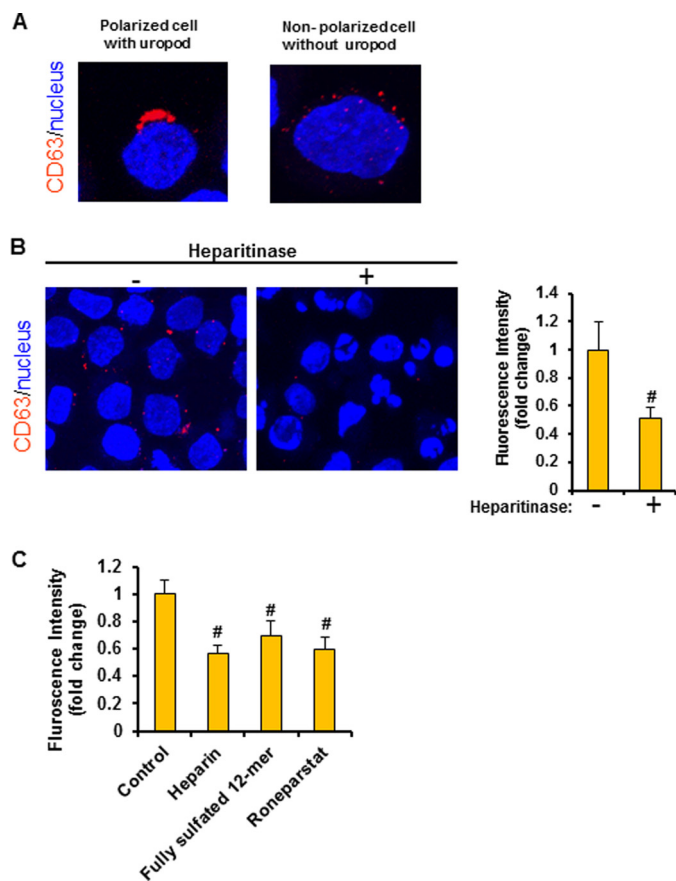
FIGURE 3. **Exosome-target cell interaction is mediated by heparan sulfate chains on target cells.** *A*, confocal microscopy analysis of polarized or non-polarized CAG myeloma cells following addition of CD63-RFP exosomes. Exosomes preferentially interact with heparan sulfate-rich uropods of polarized cells but are widely distributed on nonpolarized cells. *B*, depletion of heparan sulfate chains on myeloma cells decreases exosome-target cell interaction. RPMI-8226 cells were untreated or treated with heparitinase and washed, and their interactions with exosomes were analyzed by confocal microscopy. *Right panel*, quantitative data from a similar experiment analyzed by flow cytometry. #, $p < 0.05$ compared with no heparitinase treatment. *C*, heparin and heparin oligosaccharides inhibit exosome-target cell interaction. CD63-RFP exosomes were incubated with RPMI-8226 cells in the presence of heparin (10 μg/ml), fully sulfated heparin dodecasaccharide (12-mer) (100 μg/ml), or Roneparstat (10 μg/ml). Exosome alone is used as the control. After 2 h, exosome-target cell interaction was quantified by flow cytometry. The results are the means ± S.D. from three separate experiments. #, $p < 0.05$ compared with control.

pattern of heparan sulfate localization (Fig. 3*A*). Treatment of target cells with heparitinase prior to incubation with exosomes abolished exosome-cell interactions, thereby confirming a role for cell surface heparan sulfate chains in binding to the exosomes (Fig. 3*B*).

Because disruption of exosome-target cell interaction could be of therapeutic value, we tested whether heparin mimetics would compete against exosome and/or cell surface heparan sulfate and thereby disrupt the interaction. Two mimetics were tested, a fully sulfated 12-mer oligosaccharide (that was generated by chemoenzymatic synthesis), and Roneparstat, which is 100% *N*-acetylated and 25% glycol split. Both mimetics inhibited exosome-target cell interaction, as did heparin, the positive control for the experiment (Fig. 3*C*). Although heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effec-

tively block exosome-cell interaction. In addition, heparin 12-mers that are devoid of either *N*-sulfation, 2-*O*-sulfation, or 6-*O*-sulfation were not as effective as fully sulfated 12-mer in their ability to block exosome-cell interaction (data not shown).

Next, to determine whether fibronectin on the exosome surface was binding to heparan sulfate on target cells, we utilized reagents that would block that interaction. Fibronectin exists either in a soluble dimeric form or an insoluble, fibrillar multimeric form (15). One monomer is composed by the repeat of modular segments of 40–90 amino acids, termed type I, II, and III repeat units (15). The heparin/heparan sulfate-binding domain known as Hep-II, which is located in the type III segment, is the strongest heparin/heparan sulfate-binding site within fibronectin. When target cells were preincubated with a fragment of the fibronectin protein containing the Hep-II binding site, it greatly diminished binding of CD63-RFP exosomes as assessed by confocal microscopy and flow cytometry (Fig. 4*A*). A similar result was found when exosomes were labeled with PKH67, a lipophilic dye that incorporates within lipid membranes of vesicles (Fig. 4*A*). Next we utilized A32, an anti-fibronectin monoclonal antibody specific for the Hep-II binding domain of fibronectin that blocks binding of fibronectin to heparan sulfate (30). Preincubation of this antibody with exosomes inhibited their interaction with target cells, thereby confirming the role of the Hep-II domain in exosome-target cell interaction (Fig. 4*B*). It is important to note that antibody A32 binds to fibronectin in a heparin/heparan sulfate-dependent manner. Previous work demonstrated that heparin-induced conformational changes in fibronectin increases the binding of A32 to fibronectin (31). Thus it is possible that binding of fibronectin to heparan sulfate on the surface of exosomes increases the availability of epitopes for A32, thereby contributing to the effectiveness of A32 inhibition of exosome-cell interactions.

Interaction of Myeloma Patient-derived Exosomes with Target Cells Is Mediated by Fibronectin Binding to Heparan Sulfate—To determine whether the above *in vitro* findings are relevant in humans, exosomes from the sera of three treatment naïve multiple myeloma patients were isolated using an Exo-Quick isolation kit and further purified using anti-CD63 linked to magnetic beads. Characterization of the purified particles by nanoparticle tracking revealed that they ranged in size from 50 to 200 nm, with the majority of particles smaller than 150 nm, consistent with the size range of exosomes (Fig. 5*A*). ELISA revealed that fibronectin was present on the exosomes from all three myeloma patients examined (Fig. 5*B*). Interestingly, exosomes isolated from the serum of healthy controls also had fibronectin on their surface, indicating that the mechanism we describe may be relevant to exosomes secreted by both tumor and normal cells (data not shown). Further characterization by flow cytometry of exosomes from one of the myeloma patients demonstrated that both syndecan-1 and fibronectin were on the exosome surface (Fig. 5*C*). As was found with exosomes secreted by the CAG myeloma cell line (Fig. 1), enzymatic removal of heparan sulfate from the patient exosomes greatly diminished the amount of fibronectin associated with exosomes (Fig. 5, *D* and *E*).
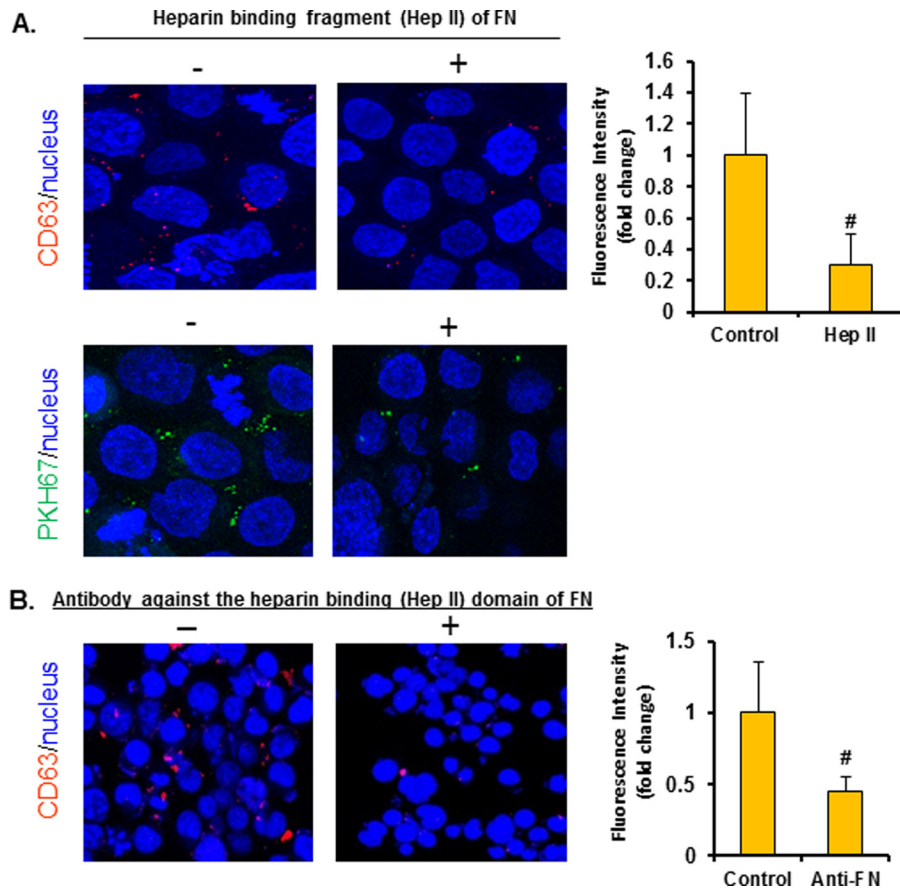
FIGURE 4. **Fibronectin on exosomes binds to heparan sulfate on target cells to mediate exosome-target cell interaction.** *A*, heparin binding fragment of the fibronectin protein inhibits exosome interaction. RPMI-8226 cells were untreated or treated with a heparin binding (Hep-II) 40-kDa fragment of purified human plasma fibronectin for 1 h, washed, and then incubated for 2 h with CD63-RFP exosomes or PKH67-labeled exosomes. The cells were then washed and fixed, and exosome interactions were analyzed by confocal microscopy. Quantitative data from a similar experiment, using CD63-RFP exosomes, were analyzed by flow cytometry (*right panel*). #, $p < 0.05$ compared with control. *B*, a monoclonal antibody (A32) that binds to the Hep-II domain of fibronectin decreases exosome-target cell interaction. CD63-RFP exosomes were preincubated with A32 antibody for 1 h and then added to RPMI-8226 cells. After 2 h, the cells were fixed and analyzed by confocal microscopy. Quantitative data from similar experiment were analyzed by flow cytometry (*right panel*). #, $p < 0.05$ compared with control. *FN*, fibronectin.

To determine whether fibronectin on exosomes from patients mediates interaction with target cells, serum exosomes were isolated, fluorescently labeled with PKH26 and incubated with cells from a human bone marrow stromal cell line. The exosomes readily interacted with stromal cells, and this was inhibited by pretreatment of exosomes with monoclonal antibody A32 that binds to and blocks the Hep-II heparin-binding domain of fibronectin (Fig. 5*F*). Together, these studies demonstrate that exosomes in the circulation of myeloma patients have fibronectin bound to their surface by heparan sulfate and that the exosomal fibronectin interacts with heparan sulfate chains on target cells to mediate the exosome-target cell interaction.

The Fibronectin-mediated Interaction of Exosomes with Target Cells Impacts Target Cell Function—We have previously demonstrated that exosomes secreted by the heparanase-high, aggressive myeloma cells promotes myeloma cell spreading and endothelial cell invasion. To explore this further and to determine whether the fibronectin-mediated interaction of exosomes with target cells impacted their function, we performed two experiments. First, an antibody array was utilized to determine whether specific signaling events were initiated in the target cells shortly after their interaction with exosomes. Exo-

somes secreted by aggressive (heparanase-high) myeloma cells were incubated with RPMI-8226 myeloma cells for 2 h, the cells were extracted, and the extracts incubated with antibody arrays to determine the level of tyrosine phosphorylation of specific molecules within key signaling pathways. The results demonstrated that cells incubated with exosomes have increased levels of phosphorylated p38 and to a lesser extent phosphorylated ERK (Fig. 6*A*). Further, to determine whether the enhanced p38 activation induced by cells is dependent on the fibronectin-heparan sulfate interactions, equal amounts of exosomes (100 μg) from control and aggressive myeloma cells were added to RPMI-8226 cells and analyzed for phosphorylated p38 by Western blotting. Fig. 6*B* shows that aggressive exosomes induces p38 activation in RPMI-8226, and this is inhibited when heparan sulfate chains are removed from RPMI8226 cells. These results demonstrate that enhanced docking of exosomes to cells through a fibronectin-heparan sulfate interaction can activate signaling pathways in myeloma cells. To determine whether the up-regulation in p38 signaling was sufficient to impact gene expression, we examined its downstream targets DKK1 and MMP-9. Analysis by PCR revealed that transcription of both DKK1 and MMP-9 were elevated in the cells following interaction with exosomes (Fig. 6*C*). In a second approach, the
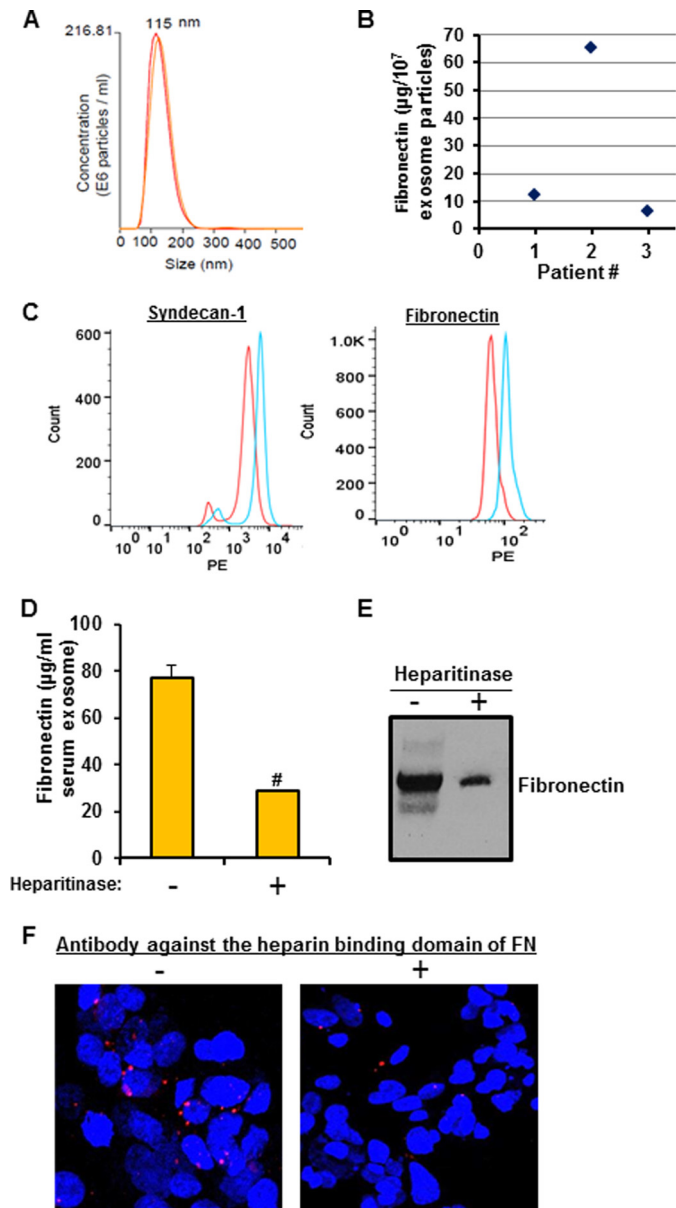
FIGURE 5. **Fibronectin on exosomes isolated from multiple myeloma patients facilitates interaction with bone marrow stromal cells.** *A*, characterization of exosomes from serum of treatment naïve multiple myeloma patients. Exosomes were purified from serum of myeloma patients by ExoQuick precipitation followed by isolation using anti-CD63 conjugated beads. Particle size was analyzed by nanoparticle tracking using a NanoSight 300. Histogram shows *two lines* representing duplicate analyses. *B*, ELISA quantification of the levels of fibronectin in exosomes isolated from serum of three myeloma patients. *C*, syndecan-1 and fibronectin are present on the surface of myeloma patient derived exosomes. Exosomes purified from the serum of myeloma patients using ExoQuick precipitation followed by anti-CD63 magnetic bead isolation were subjected to flow cytometry analysis using an affinity-purified polyclonal goat anti-syndecan-1 IgG antibody (*blue*) or mouse monoclonal PE-conjugated anti-fibronectin antibody (*blue*). Normal goat IgG and mouse IgG1 isotype were used as control (*red*), respectively. Note that syndecan-1 (core protein) was detected on the surface of exosomes only after removal of heparan sulfate chains by heparitinase treatment to expose the epitope. *D* and *E*, removal of heparan sulfate chains removes most of the fibronectin from exosomes isolated from the serum of myeloma patients. Exosomes isolated from patient serum were either not treated or treated with heparitinase, and fibronectin levels were quantified by ELISA (*D*) and compared by Western blot (*E*). #, $p < 0.05$ compared with heparitinase untreated exosomes. *F*, A32 antibody against the Hep-II domain of fibronectin blocks the interaction of patient-derived exosomes with bone marrow stromal cells. Exosomes isolated from the serum of a myeloma

impact of exosome-endothelial cell interaction on endothelial cell invasion was examined. Exosomes were preincubated with antibody to the Hep-II heparin-binding domain of fibronectin, and their ability to promote human umbilical vein endothelial cell invasion through Matrigel-coated chambers was compared with exosomes not exposed to antibody. The results indicate that blocking of fibronectin with the antibody significantly inhibited endothelial invasion (Fig. 6*D*). Together, these studies indicate that fibronectin-mediated interaction of exosomes with target cells can significantly impact target cell behavior.

Discussion

This work demonstrates a novel mechanism through which exosomes secreted by cancer cells interact with target cells. The data indicate that heparan sulfate on the exosome surface captures fibronectin, and when a target cells is encountered, the exosomal fibronectin binds to heparan sulfate proteoglycans present on the target cell surface (Fig. 7). This model is supported by studies utilizing exosomes from both myeloma cell lines and myeloma patient sera. Although we cannot verify that all the vesicles isolated from patients were derived from tumor cells, a relatively high number of the isolated exosomes were CD138-positive (Fig. 5), in line with the fact that most myeloma cells express high levels of this marker (32). In addition, most of the patient-derived exosomes had fibronectin on their surface that could be removed by treatment with heparan sulfate degrading enzyme. Further, blocking the Hep-II domain with antibodies inhibited binding of the patient exosomes to target cells (Fig. 5). Together, these data support the notion that fibronectin on circulating exosomes from myeloma patients mediates their binding to heparan sulfate on target cells. Because of its abundance on cell surfaces, within the extracellular matrix and in the serum, we speculate that fibronectin is not restricted to tumor-derived exosomes but is present on the surface of exosomes derived from many cell types in both normal and pathological settings. In addition, it seems likely that exosome-fibronectin interaction may also facilitate exosome binding to extracellular matrix by binding to collagens or heparan sulfate trapped within the matrix. This is important because tumor-derived exosomes have been shown to bind components of the extracellular matrix where they can promote matrix degradation and tumor cell invasion and motility (33).

Interestingly, and in support of our finding of fibronectin on the surface of exosomes, it was recently demonstrated that exosomes present in blood plasma of normal human subjects can be isolated on heparin affinity beads (34), clearly demonstrating that a ligand for heparan sulfate exists on the surface of circulating exosomes. Moreover, heparin has been shown to directly interact with extracellular vesicles and heparin inhibits vesicle binding to target cells (11, 14). In another study, exogenous heparin or treatment of cells with heparan sulfate degrading enzymes reduced exosome-target cell interaction by ~50% (12). However, in contrast to our findings that point to a role for

patient were labeled with PKH26 (*red*) dye, preincubated with or without A32 antibody for 1 h, and allowed to bind HS-5 human bone marrow stromal cells. After 2 h cells were fixed, and images were analyzed by confocal microscopy. *FN*, fibronectin.
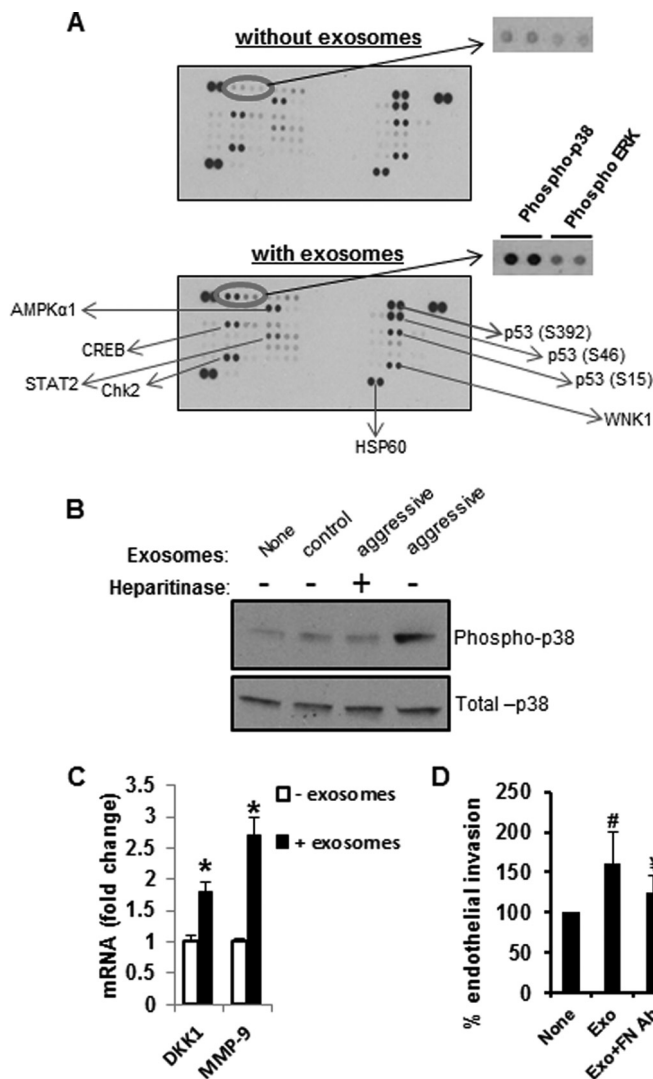
FIGURE 6. **Exosome-target cell interaction mediated by fibronectin impacts cell behavior.** *A*, exosome interaction with RPMI-8226 cells activates p38 and ERK signaling. An antibody array that simultaneously examines the phosphorylation levels of 43 different protein kinases was utilized to determine what signaling pathways were activated when myeloma-derived exosomes interacted with target cells. RPMI-8226 cells were incubated with or without exosomes isolated from aggressive myeloma cells (CAG cells expressing high heparanase), cell lysates were exposed to membranes, and the membranes were probed with a phosphotyrosine specific antibody. Phosphorylated p38 and ERK (*circled*) were enhanced in cells incubated with the exosomes. The different phospho kinases that are activated in RPMI-8226 cells independent of the addition of exosomes are shown by *arrows*. *Duplicate dots* at the corners represent phosphotyrosine positive controls. *B*, RPMI-8226 cells were treated with exosomes (100 μg/ml) secreted by control or aggressive myeloma cells for 20 min and analyzed for phosphorylated p38 by Western blot. Total p38 serves as the loading control. To determine the role of cell surface heparan sulfate in mediating exosome-induced signaling, RPMI-8266 cells were treated with bacterial heparitinase for 2 h prior to the addition of exosomes from aggressive myeloma cells. *C*, MMP-9 and DKK1, two downstream target genes of activated p38, are up-regulated following the interaction of myeloma-derived exosomes with the RPMI 8226 cells. RPMI 8266 myeloma cells were incubated with or without exosomes, and DKK1 and MMP-9 mRNA expression in these cells was assessed using real time PCR and normalized using actin expression. *, $p < 0.05$ compared with no exosome addition. *D*, 10 μg of CD63-RFP exosomes from aggressive CAG cells were added to endothelial cells in the presence or absence of A32 antibody, and the number of cells that invaded through the Matrigel-coated chamber overnight was determined. The data are means ± S.D. from three separate experiments. #, $p < 0.01$ compared with no exosome addition; ¥, $p < 0.05$ compared with exosomes only.
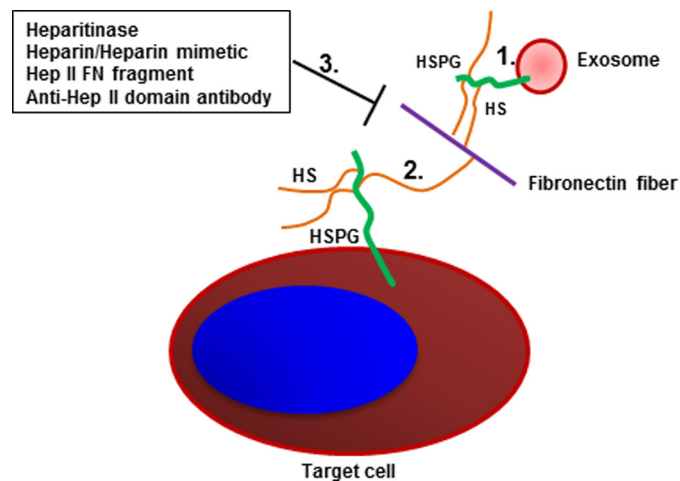

FIGURE 7. **Model of exosome-target cell interaction mediated by fibronectin.** *Step 1*, fibronectin (*FN*) is captured on the surface of exosomes by heparan sulfate proteoglycans (*HSPG*). *Step 2*, fibronectin, on exosomes binds to heparan sulfate (*HS*) on the surface of the target cell. *Step 3*, removal of exosome heparan sulfate or cell surface heparan sulfate with heparitinase, addition of exogenous heparin or heparin mimetics, addition of Hep-II domain-containing fragment of fibronectin, or exposure to antibody against the Hep-II domain of fibronectin dramatically diminishes exosome interaction with target cells.

exosomal fibronectin in exosome-target cell interactions, these prior studies failed to identify the ligand present on the exosome that was interacting with heparan sulfate on the cell surface.

Although fibronectin has been detected by mass spectroscopy as cargo within exosomes from many cell sources (35, 36), to our knowledge, our data provide the first evidence that fibronectin is mediating exosome-target cell interaction. Fibronectin has been shown to be present on the surface of exosomes secreted by trophoblasts where it associates with macrophages and induces their expression of IL-1β (37). This occurs without exosome internalization and is likely mediated through the interaction of exosome fibronectin with integrin surface receptors on the macrophages. In addition, it was recently demonstrated that HT1080 fibrosarcoma cells deposit exosomes to the substrata that are assessable to the basal cell surface. These exosomes have surface fibronectin that helps support cell adhesion and accelerate cell migration (38). In this case, it was demonstrated that ~50% of the fibronectin could be displaced from exosomes by incubation with the RGD peptide, indicating that at least some of the fibronectin was bound to the exosome via integrins. Similarly, fibronectin was found on the surface of exosomes isolated from *ex vivo* cultured 4T1 breast cancer cells, and those exosomes could stimulate tumor cell migration via activation of integrins (39). However, none of the above studies examined whether the fibronectin on the exosomes being studied associated with heparan sulfate present either on the exosome or on the cell surface. Interactions between fibronectin and heparan sulfate could explain why only half the fibronectin was displaced from the fibrosarcoma-derived exosomes by the RGD peptide (38). Interestingly, although we did not explore whether integrins were involved in the targeting of myeloma-derived exosomes to cells, we found that removal of heparan sulfate from exosomes did remove most of the exosome associated fibronectin (Figs. 2 and 5). This

suggests that heparan sulfate is responsible for binding most of the fibronectin to the surface of these myeloma-derived exosomes. Notably, removal of heparan sulfate from target cells only reduced exosome binding by ~50% (Fig. 3), and interfering with the Hep-II domain of fibronectin (Fig. 4) also only partially inhibited binding of exosomes. This raises the possibility that integrin receptors on target cells may also contribute to the fibronectin-mediated targeting.

Our results indicate that heparan sulfate plays a dual role in the interaction between exosomes and cells, first by capturing fibronectin on the exosome surface and then through interaction of exosome fibronectin with heparan sulfate on the surface of cells. Because heparan sulfate is ubiquitously expressed on cell surfaces, the mechanism we describe here is likely not mediating targeting of exosomes to specific cell types. Rather, this may be a general mechanism that mediates initial binding of exosomes to most cells. However, some specificity of exosome binding to cells could be conferred through the structure of cell surface heparan sulfate chains. For example, it was demonstrated that cells lacking heparan sulfate 2-*O*- or -*N*-sulfation exhibited reduced exosome binding compared with controls (13). It was previously demonstrated that after binding of exosomes to cells, cell surface heparan sulfate proteoglycans were internalized by cells thereby mediating exosome uptake (13). Thus cell surface heparan sulfate proteoglycans appear to be internalizing receptors for exosomes rather than just cell surface attachment sites. Although the mechanism of release of exosome cargo within cells is not clear, once internalized, exosomes may fuse with the delimiting membrane of the endocytic compartment and deliver the cargo to the cytosol of target cells (40). It is not known whether heparan sulfate proteoglycans play a role in exosome cargo release.

In regard to heparan sulfate on exosomes, it has been shown that the syndecan heparan sulfate proteoglycans, through formation of a molecular complex with syntenin and ALIX, help drive exosome formation (41). We found that heparanase stimulated exosome secretion in myeloma cells (9), and further studies by Roucourt *et al.* (42) recently confirmed our findings and demonstrated that heparanase, by trimming the heparan sulfate chains of syndecan, activates the syndecan-syntenin-ALIX pathway. Interestingly, heparanase causes more syndecan-1 to be recruited to exosomes (9, 42), and in the present work, we discovered that this results in a greater amount of fibronectin associating with the exosome surface leading to enhanced exosome-target cell interaction (Fig. 1). Thus our current findings demonstrate that the importance of the heparanase/syndecan axis in regulating the biology of exosomes is not restricted solely to exosome biogenesis and cargo content but also impacts exosome-target cell interaction, a key step in the ability of exosomes to regulate cell behavior.

Although we find that heparan sulfate on myeloma-derived exosomes plays an important role in their interaction with target cells, this may not be a universal mechanism used by all exosomes. For example, using exosomes isolated from the U-87 MG glioblastoma cell line, it was concluded that although both syndecan and glypican proteoglycans were associated with the exosomes, heparan sulfate present on the exosome was not involved in interactions with target U-87 MG cells (12). This does not preclude the possibility that fibronectin is involved, because fibronectin in this setting, rather than being bound by heparan sulfate chains, could be attached to exosomes predominantly via integrins present on the exosome surface. Another possibility is that myeloma cell-derived exosomes have a higher level of heparan sulfate and/or fibronectin on their surface that drives the exosome-cell interaction as compared with the glioblastoma-derived exosomes. This would be consistent with our observation that exosomes having low levels of heparan sulfate and fibronectin do not interact with cells as avidly as exosomes having higher levels of these molecules (Fig. 1). This suggests, at least in the case of the myeloma cells, that the capacity of exosomes to interact with target cells is regulated by the amount of heparan sulfate on the exosome surface.

We demonstrate that the exosome-target cell interaction can be blocked by enzymatic removal of heparan sulfate from either the exosome or target cell, by exposure to heparin/heparin analogues that interfere with heparan sulfate/fibronectin interactions, by antibody against the Hep-II protein domain within fibronectin that binds to heparan sulfate, or by addition of excess fragments of the fibronectin protein that contain the Hep-II domain. These findings not only support our model of fibronectin-heparan sulfate-mediated interaction of exosomes with cells, but also expose multiple ways in which that interaction could be attacked in patients for therapeutic benefit. Of particular importance is the finding that heparin, a synthetic fully sulfated 12-mer heparin mimetic, and heparin-derived Roneparstat are all capable of significantly inhibiting targeting of exosomes to cells. Importantly, unlike heparin, both the 12-mer mimetic and Roneparstat lack significant anticoagulant activity and thus can be delivered to patients at high doses, thereby disrupting or preventing exosome-target cell interactions. These findings reveal a previously undescribed mechanism of action for this class of drugs and provide further rationale for their investigation in clinical trials.

References

1. Szalat, R., and Munshi, N. C. (2015) Genomic heterogeneity in multiple myeloma. *Curr. Opin. Genet. Dev.* **30,** 56–65
2. Witwer, K. W., Buzás, E. I., Bemis, L. T., Bora, A., Lässer, C., Lötvall, J., Nolte-'t Hoen, E. N., Piper, M. G., Sivaraman, S., Skog, J., Théry, C., Wau-

ben, M. H., and Hochberg, F. (2013) Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J. Extracell. Vesicles* **2,** 10.3402/jev.v3402i3400.20360

3. Peinado, H., Lavotshkin, S., and Lyden, D. (2011) The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. *Semin. Cancer Biol.* **21,** 139–146

4. Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., García-Santos, G., Ghajar, C., Nitadori-Hoshino, A., Hoffman, C., Badal, K., Garcia, B. A., Callahan, M. K., Yuan, J., Martins, V. R., Skog, J., Kaplan, R. N., Brady, M. S., Wolchok, J. D., Chapman, P. B., Kang, Y., Bromberg, J., and Lyden, D. (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* **18,** 883–891

5. Zhang, H. G., and Grizzle, W. E. (2014) Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. *Am. J. Pathol.* **184,** 28–41

6. Kharaziha, P., Ceder, S., Li, Q., and Panaretakis, T. (2012) Tumor cell-derived exosomes: a message in a bottle. *Biochim. Biophys. Acta* **1826,** 103–111

7. Raimondi, L., De Luca, A., Amodio, N., Manno, M., Raccosta, S., Taverna, S., Bellavia, D., Naselli, F., Fontana, S., Schillaci, O., Giardino, R., Fini, M., Tassone, P., Santoro, A., De Leo, G., Giavaresi, G., and Alessandro, R. (2015) Involvement of multiple myeloma cell-derived exosomes in osteoclast differentiation. *Oncotarget* **6,** 13772–13789

8. Umezu, T., Tadokoro, H., Azuma, K., Yoshizawa, S., Ohyashiki, K., and Ohyashiki, J. H. (2014) Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1. *Blood* **124,** 3748–3757

9. Thompson, C. A., Purushothaman, A., Ramani, V. C., Vlodavsky, I., and Sanderson, R. D. (2013) Heparanase regulates secretion, composition, and function of tumor cell-derived exosomes. *J. Biol. Chem.* **288,** 10093–10099

10. Sarrazin, S., Lamanna, W. C., and Esko, J. D. (2011) Heparan sulfate proteoglycans. *Cold Spring Harb. Perspect. Biol.* **3,** a004952

11. Atai, N. A., Balaj, L., van Veen, H., Breakefield, X. O., Jarzyna, P. A., Van Noorden, C. J., Skog, J., and Maguire, C. A. (2013) Heparin blocks transfer of extracellular vesicles between donor and recipient cells. *J. Neurooncol.* **115,** 343–351

12. Christianson, H. C., and Belting, M. (2013) Heparan sulfate proteoglycan as a cell-surface endocytosis receptor. *Matrix Biol.* **35,** 51–55

13. Christianson, H. C., Svensson, K. J., van Kuppevelt, T. H., Li, J. P., and Belting, M. (2013) Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 17380–17385

14. Di Noto, G., Chiarini, M., Paolini, L., Mazzoldi, E. L., Giustini, V., Radeghieri, A., Caimi, L., and Ricotta, D. (2014) Immunoglobulin free light chains and GAGs mediate multiple myeloma extracellular vesicles uptake and secondary NFκB nuclear translocation. *Front. Immunol.* **5,** 517

15. Carpentier, M., Denys, A., Allain, F., and Vergoten, G. (2014) Molecular docking of heparin oligosaccharides with Hep-II heparin-binding domain of fibronectin reveals an interplay between the different positions of sulfate groups. *Glycoconj. J.* **31,** 161–169

16. Tumova, S., Woods, A., and Couchman, J. R. (2000) Heparan sulfate chains from glypican and syndecans bind the Hep II domain of fibronectin similarly despite minor structural differences. *J. Biol. Chem.* **275,** 9410–9417

17. Woods, A., Longley, R. L., Tumova, S., and Couchman, J. R. (2000) Syndecan-4 binding to the high affinity heparin-binding domain of fibronectin drives focal adhesion formation in fibroblasts. *Arch. Biochem. Biophys.* **374,** 66–72

18. Dalton, B. A., McFarland, C. D., Underwood, P. A., and Steele, J. G. (1995) Role of the heparin binding domain of fibronectin in attachment and spreading of human bone-derived cells. *J. Cell Sci.* **108,** 2083–2092

19. Børset, M., Hjertner, O., Yaccoby, S., Epstein, J., and Sanderson, R. D. (2000) Syndecan-1 is targeted to the uropods of polarized myeloma cells where it promotes adhesion and sequesters heparin-binding proteins. *Blood* **96,** 2528–2536

20. Purushothaman, A., Chen, L., Yang, Y., and Sanderson, R. D. (2008) Hepa-

ranase stimulation of protease expression implicates it as a master regulator of the aggressive tumor phenotype in myeloma. *J. Biol. Chem.* **283,** 32628–32636

21. Purushothaman, A., and Toole, B. P. (2014) Serglycin proteoglycan is required for multiple myeloma cell adhesion, in vivo growth, and vascularization. *J. Biol. Chem.* **289,** 5499–5509

22. Rajkumar, S. V., Dimopoulos, M. A., Palumbo, A., Blade, J., Merlini, G., Mateos, M. V., Kumar, S., Hillengass, J., Kastritis, E., Richardson, P., Landgren, O., Paiva, B., Dispenzieri, A., Weiss, B., LeLeu, X., Zweegman, S., Lonial, S., Rosinol, L., Zamagni, E., Jagannath, S., Sezer, O., Kristinsson, S. Y., Caers, J., Usmani, S. Z., Lahuerta, J. J., Johnsen, H. E., Beksac, M., Cavo, M., Goldschmidt, H., Terpos, E., Kyle, R. A., Anderson, K. C., Durie, B. G., and Miguel, J. F. (2014) International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol.* **15,** e538–e548

23. Meares, G. P., Liu, Y., Rajbhandari, R., Qin, H., Nozell, S. E., Mobley, J. A., Corbett, J. A., and Benveniste, E. N. (2014) PERK-dependent activation of JAK1 and STAT3 contributes to endoplasmic reticulum stress-induced inflammation. *Mol. Cell Biol.* **34,** 3911–3925

24. Ritchie, J. P., Ramani, V. C., Ren, Y., Naggi, A., Torri, G., Casu, B., Penco, S., Pisano, C., Carminati, P., Tortoreto, M., Zunino, F., Vlodavsky, I., Sanderson, R. D., and Yang, Y. (2011) SST0001, a chemically modified heparin, inhibits myeloma growth and angiogenesis via disruption of the heparanase/syndecan-1 axis. *Clin. Cancer Res.* **17,** 1382–1393

25. Liu, J., and Linhardt, R. J. (2014) Chemoenzymatic synthesis of heparan sulfate and heparin. *Nat. Prod. Rep.* **31,** 1676–1685

26. Kelly, T., Miao, H. Q., Yang, Y., Navarro, E., Kussie, P., Huang, Y., MacLeod, V., Casciano, J., Joseph, L., Zhan, F., Zangari, M., Barlogie, B., Shaughnessy, J., and Sanderson, R. D. (2003) High heparanase activity in multiple myeloma is associated with elevated microvessel density. *Cancer Res.* **63,** 8749–8756

27. Purushothaman, A., Uyama, T., Kobayashi, F., Yamada, S., Sugahara, K., Rapraeger, A. C., and Sanderson, R. D. (2010) Heparanase-enhanced shedding of syndecan-1 by myeloma cells promotes endothelial invasion and angiogenesis. *Blood* **115,** 2449–2457

28. Yang, Y., Macleod, V., Bendre, M., Huang, Y., Theus, A. M., Miao, H. Q., Kussie, P., Yaccoby, S., Epstein, J., Suva, L. J., Kelly, T., and Sanderson, R. D. (2005) Heparanase promotes the spontaneous metastasis of myeloma cells to bone. *Blood* **105,** 1303–1309

29. Yang, Y., Ren, Y., Ramani, V. C., Nan, L., Suva, L. J., and Sanderson, R. D. (2010) Heparanase enhances local and systemic osteolysis in multiple myeloma by upregulating the expression and secretion of RANKL. *Cancer Res.* **70,** 8329–8338

30. Underwood, P. A., Dalton, B. A., Steele, J. G., Bennett, F. A., and Strike, P. (1992) Anti-fibronectin antibodies that modify heparin binding and cell adhesion: evidence for a new cell binding site in the heparin binding region. *J. Cell Sci.* **102,** 833–845

31. Hubbard, B., Buczek-Thomas, J. A., Nugent, M. A., and Smith, M. L. (2014) Heparin-dependent regulation of fibronectin matrix conformation. *Matrix Biol.* **34,** 124–131

32. Bayer-Garner, I. B., Sanderson, R. D., Dhodapkar, M. V., Owens, R. B., and Wilson, C. S. (2001) Syndecan-1 (CD138) immunoreactivity in bone marrow biopsies of multiple myeloma: shed syndecan-1 accumulates in fibrotic regions. *Mod. Pathol.* **14,** 1052–1058

33. Mu, W., Rana, S., and Zöller, M. (2013) Host matrix modulation by tumor exosomes promotes motility and invasiveness. *Neoplasia* **15,** 875–887

34. Balaj, L., Atai, N. A., Chen, W., Mu, D., Tannous, B. A., Breakefield, X. O., Skog, J., and Maguire, C. A. (2015) Heparin affinity purification of extracellular vesicles. *Sci. Rep.* **5,** 10266

35. Marimpietri, D., Petretto, A., Raffaghello, L., Pezzolo, A., Gagliani, C., Tacchetti, C., Mauri, P., Melioli, G., and Pistoia, V. (2013) Proteome profiling of neuroblastoma-derived exosomes reveal the expression of proteins potentially involved in tumor progression. *PLoS One* **8,** e75054

36. Melo, S. A., Luecke, L. B., Kahlert, C., Fernandez, A. F., Gammon, S. T., Kaye, J., LeBleu, V. S., Mittendorf, E. A., Weitz, J., Rahbari, N., Reissfelder, C., Pilarsky, C., Fraga, M. F., Piwnica-Worms, D., and Kalluri, R. (2015) Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* **523,** 177–182

37. Atay, S., Gercel-Taylor, C., and Taylor, D. D. (2011) Human trophoblast-derived exosomal fibronectin induces pro-inflammatory IL-1beta production by macrophages. *Am. J. Reprod. Immunol.* **66,** 259–269

38. Sung, B. H., Ketova, T., Hoshino, D., Zijlstra, A., and Weaver, A. M. (2015) Directional cell movement through tissues is controlled by exosome secretion. *Nat. Commun.* **6,** 7164

39. Deng, Z., Cheng, Z., Xiang, X., Yan, J., Zhuang, X., Liu, C., Jiang, H., Ju, S., Zhang, L., Grizzle, W., Mobley, J., Roman, J., Miller, D., and Zhang, H. G. (2012) Tumor cell cross talk with tumor-associated leukocytes leads to induction of tumor exosomal fibronectin and promotes tumor progression. *Am. J. Pathol.* **180,** 390–398

40. Raposo, G., and Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* **200,** 373–383

41. Baietti, M. F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortere, F., Coomans, C., Vermeiren, E., Zimmermann, P., and David, G. (2012) Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat. Cell Biol.* **14,** 677–685

42. Roucourt, B., Meeussen, S., Bao, J., Zimmermann, P., and David, G. (2015) Heparanase activates the syndecan-syntenin-ALIX exosome pathway. *Cell Res.* **25,** 412–428